NEURODEGENERATION AND NEUROGENESIS IN MOUSE MODELS OF AGING AND ALZHEIMER'S DISEASE

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TABLE OF CONTENTS

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SUMMARY

As the proportion of senior citizens gradually increases, the behavioral changes that occur with normal aging and as a consequence of Alzheimer's disease (AD) will afflict many of us in the future. Aging is the major risk factor for AD, and pathological changes that occur in AD are superimposed upon normal aging alterations. Thus, to understand etiologies and mechanisms of AD it is important to distinguish normal aging from disease processes. In search of structural parameters, which could correlate with the behavioral changes during normal aging and AD, the discovery of neural progenitor cells and neurogenesis in the adult mammalian brain has received much attention. Furthermore, advances in stem cell techniques have raised the possibility for neuronal replacement strategies in neurodegenerative diseases such as AD. With progresses in mouse genetics and the identification of genes linked to AD it has become possible to generate transgenic mouse models that mimic key aspects of AD pathology. Studies involving such mouse models have identified beta-amyloid peptide (Aβ), the main component of amyloid plaques, as an important factor in the pathophysiology of AD. However, no general consensus exists about the mechanism by which Aβ exerts its detrimental effects. The research described herein addresses key questions regarding (i) neurogenesis and its modulation in the aging mouse brain, (ii) the impact of cerebral amyloidosis on neurodegeneration and neurogenesis in a transgenic mouse model of AD, and (iii) the application of a promising anti-Aβ immunotherapy in this transgenic mouse model.

In a first study, we have examined the effect of aging on neurogenesis in the dentate gyrus of C57BL/6 (B6) mice. We used the B6 line because it is one of the best characterized mouse strains in neuroscience, and because it was shown to be relatively resistant to age-related structural brain changes. Our results revealed a striking decrease in neurogenesis due to an age-related reduction in neuronal proliferation. Interestingly, this decrease was observed until late adulthood with no further decline with aging. Stimulated by recent findings that caloric restriction (CR) might increase neurogenesis in young rodents, the potential of CR to postpone the age-related decrease in neurogenesis was tested. However, results revealed no impact of CR on hippocampal neurogenesis. Instead, a survival-promoting effect of CR on newborn glial cells in the hilar region was observed.

In a second study, the impact of cerebral amyloidosis on neurodegeneration was studied using a recently generated murine model of AD, the APP23 mouse. This transgenic line overexpresses a mutated human form of the amyloid precursor protein (APP), develops amyloid plaques, and shows cognitive impairments with aging. Stereological estimation revealed a modest but significant age-related neuron loss in the neocortex of APP23 mice. This observation is consistent with the appearance of plaque-associated apoptotic and necrotic neurons in aged APP23 mice. Encouraged by recent reports that demonstrated neocortical neurogenesis after targeted apoptosis, we examined neurogenesis in the neocortex of APP23 mice with a high amyloid burden. However, no evidence for neocortical neurogenesis, both in young and aged APP23 mice, was found. In contrast, we found a fivefold increase in gliogenesis in aged transgenic mice when compared to littermate controls.

During the last few years several therapeutic strategies have been proposed for treating AD, and some of them have entered clinical trials. For example, it has been suggested that vaccination with Aβ reduces cerebral amyloidosis and protects against cognitive deficits in different mouse models of AD. Thus, in a third study, we investigated the effect of passive immunization in the APP23 mouse, a model that exhibits amyloid plaques as well as cerebral amyloid angiopathy (CAA), similar to that observed in the human AD brain. Our results showed significant clearance of diffuse amyloid and reductions in the levels of the highly fibrillogenic $A\beta_{42}$. However, immunized mice exhibited a robust increase in the frequency and severity of CAA-associated cerebral hemorrhages compared to non-vaccinated APP23 controls. Together with the neuroinflammatory side effects recently observed in human trials, our results further stress the need for a better understanding of the basic mechanisms involved in antibody-mediated Aβ clearance.

1. INTRODUCTION

1.1 BRAIN AGING

A common characteristic of aging is the progressive decrease in physiological capacity once the reproductive phase of life is over (Sohal and Weindruch, 1996). Brain aging is accompanied by biochemical and structural alterations that have been linked to age-related cognitive changes. Although cognitive abilities tend to decline more slowly than do physical skills, some level of cognitive impairment is expected as aging progresses (Albert et al., 1987). While in some people aging hardly affects cognition, others experience a significant decline in cognitive function, particularly in relation to memory skills (Rowe and Kahn, 1987; Rapp and Amaral, 1992). These individual differences in aging have become important in the study of genetic, cellular, morphological and environmental factors involved in aging. (Hayflick, 2000; Kirkwood and Austad, 2000; Martin and Oshima, 2000; Hekimi and Guarente, 2003; Longo and Finch, 2003). Furthermore, the surge in brain aging studies has been driven by the progressive rise in the worldwide life expectancy. According to the WHO, there are currently 600 million people over age 60, this number will double by 2025 and will reach two billion by 2050, the majority of them in the developing world. Given that aging is the major risk factor for the development of neuro-degenerative disorders such as Alzheimer's disease (AD), research on brain aging is of pivotal importance and will hopefully results in the ease of the social and economic impact of the expected exponential increase of age-related neurodegenerative diseases.

1.1.1 Mechanisms and modulation of the aging process

Aging is often considered to be a side effect of optimizing an organism's evolutionary fitness (Austad, 1997). That is, we age because a variety of gene actions escape the natural selection process. It has been shown that senescence does not contribute significantly to mortality in the wild. More important are hazards such as infection, predation, starvation or cold. This allows a wide range of alleles with late deleterious effects to accumulate (Kirkwood and Austad, 2000). The impact of genetic factors on the aging process has received much attention due to the identification of genes that regulate longevity and cell viability in animal models ranging from

yeast to mice (Kenyon et al., 1993; Lin et al., 1998; Kaeberlein et al., 1999; Migliaccio et al., 1999). Recently, some of these genes have been associated with the insulin or insulin-like signaling pathway, implicating hormones as regulators of the aging process (Holzenberger et al., 2003; Tatar et al., 2003). A recurrent feature of these mutants is their increased resistance to environmental stressors such as reactive oxygen species (ROS) or UV-irradiation. This implicates ROS and the corresponding response to oxidative stress as key aging factors (‚free-radical theory' of aging) (Sohal and Weindruch, 1996). Oxidative damage to DNA, protein, and lipids has indeed been demonstrated to occur with aging (Fraga et al., 1990; Stadtman, 1992; Liu et al., 2002).

Almost 70 years ago, it was first noted that food restriction extends the life span of rodents (McCay et al., 1935). This longevity results from the limitation of total calories derived from carbohydrates, fats, or proteins to a level of 25% to 60% below that of control animals fed ad libitum (Richardson, 1985; Weindruch et al., 1986). To the present day, caloric restriction has remained the only treatment that consistently extends the mean and maximum life span in a remarkable range of organisms (Weindruch et al., 1988). Emerging data suggests that this effect may also apply to nonhuman primates (Lane et al., 2001). CR delays a wide spectrum of diseases in animals, such as kidney diseases, autoimmune diseases, and diabetes (Koubova and Guarente, 2003). The impact of CR on brain physiology has been debated in the past, but a prevention of age-associated decline in psychomotor and spatial memory tasks has been reported (Ingram et al., 1987; Stewart et al., 1989). In addition, CR protects neurons from excitotoxic-induced degeneration in mouse models of neurodegenerative diseases such as, Huntington's disease, Parkinson's disease and AD (Bruce-Keller et al., 1999; Duan and Mattson, 1999; Zhu et al., 1999). Epidemiological studies have also suggested that individuals with low caloric intake may have reduced risk for Parkinson's disease and AD (Logroscino et al., 1996; Grant, 1999; Mayeux et al., 1999).

Despite the extensive physiological characterization of this caloric regimen, the molecular basis for the slowing of the aging process and age-related diseases remains to be fully understood. A leading hypothesis is that CR reduces oxidative damage generated by ROS produced during respiration (Lee and Yu, 1990). Indeed, it has been shown that CR retards the age-associated cellular accumulation of oxidatively damaged molecules such as lipids, proteins, and DNA

(Matsuo et al., 1993; Sohal and Dubey, 1994; Dubey et al., 1996). This is probably not achieved by a reduction in metabolism (McCarter and Palmer, 1992), suggesting that enhanced antioxidant defenses or repair mechanisms are involved (Armeni et al., 1998). Most interestingly, CR has been recently reported to enhance neurogenesis in young rodents (Lee et al., 2000; Lee et al., 2002b). It has been suggested that an increase in neurotrophic factors in CR animals might trigger survival of newborn neurons (Lee et al., 2002a). These findings are of interest given that neurogenesis has not only been implicated in memory and learning processes, but has also been shown to decrease with aging (Kuhn et al., 1996; Shors et al., 2001).

1.1.2 Neurodegeneration in the aging brain

The magnitude of structural changes that occur during normal brain aging, and how these changes correlate with cognitive decline has long been a subject of debate. Existing data about the effect of age on synapses are inconclusive. Whereas some studies reported no effect of aging on synapse number (Cragg, 1975; Tigges et al., 1996), other investigations have found an age-related decline in synapse number (Masliah et al., 1993; Peters et al., 1998). In contrast, consensus exists about decreases in specific neurotransmitter levels and the number of neurotransmitter receptors with aging. The marked loss of dopamine in the aged monkey cortex has been associated with decreased working memory performance (Arnsten, 1999). Furthermore, reductions in levels of acetylcholine, norepinephrine, and serotonin have been reported with aging, whereas glutamate, the primary cortical neurotransmitter appears not to be affected by age (Wenk et al. 1989; Beal et al., 1991; Arnsten, 1999). In addition, aging affects the myelin sheath of nerve fibers and leads to a robust fiber loss (Peters and Sethares, 2002; Marner et al., 2003). Until recently, it was widely accepted that neuron loss was an inevitable result of normal aging. Several studies performed prior to the mid 80‘s reported substantial age-related decreases in neuron number of the cortex and most hippocampal areas (Brody, 1955; Dayan, 1970; Coleman and Flood, 1987). With the development of more accurate and unbiased morphometric procedures for estimating neuron numbers, this view has been modified over the last years (Gundersen et al., 1988; West, 1993). Nowadays, it is well accepted, that in the human neocortex only a very mild age-related decline in neuron number occurs (Pakkenberg and Gundersen, 1997). Similarly, most subfields of the human hippocampus, including the granule cells of the dentate gyrus, show no decrease in neuron number with aging

(West, 1993; West et al., 1994). Such studies have been extended to rodents, and the age-related neuron loss is even more limited in these species (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Calhoun et al., 1998a). However, more recently it has been shown that the adult human brain can generate new neurons throughout life (Eriksson et al., 1998; Zhao et al., 2003). This newborn cells mature into functional neurons and are integrated into existing circuitries in the adult mammalian brain (van Praag et al., 2002). Thus the maintenance of total neuron number may be reviewed as a continuous death and birth of neurons. Studies on age-associated changes in neuron number will also have to account for the rate at which newborn neurons are replacing dying ones.

1.1.3 Application of unbiased stereology to neural systems

The quantitative assessment of neurodegeneration depends on using appropriate morphometric techniques to estimate structural changes. Some examples include whether numbers of neurons in different parts of the brain decline with age, and whether losses of neurons are indicators of the severity of a disease. Two main strategies have been used for quantification of neural structures: Assumption-based and design-based techniques. The aforementioned dogma, that normal aging is accompanied by massive nerve cell loss, had been established using assumption-based techniques. Two design characteristics in assumption-based morphometry account for this erroneous dogma: 1. The standard method for identifying cells for quantification was based on cellular profiles. 2. The investigators were measuring neuron density in a given structure, not total neuron number:

1. Assumption-based morphometry has long recognized that simple profile-based counts could not be directly correlated to the total number of neurons in a given region. For instance an increase in the number of neuronal profiles may reflect an increase in cell size, because larger neurons will appear in a higher number of sections and will thus have an increased probability of being sampled. Abercrombie was the first to introduce geometrical principles to overcome these type of problems (Abercrombie, 1946). He established correction factors for the conversion of the 2-D profile of a neuron to 3-D based on the assumption that neurons are spherical, and using standard geometric formulas to calculate its height. Although this method has coped with some of the sources of bias resulting from variability in cell size, shape, and orientation, additional

correction factors had to be introduced (West, 1993). The problem of using such correction factors is that they require information that is often difficult to estimate. As a consequence, correction factors introduce systematic error to morphometric estimates.

2. The assumption that neuron density is a reliable measure for the total number of neurons is problematic. Differences in density may not only reflect changes in number of cells, but also differences in the volume of the brain region. One striking observation was, that brain tissue from younger subjects shrinks substantially more than tissue from older subjects during tissue processing (Haug et al., 1984). The consequence is that neuronal densities are higher in younger brains when compared to older ones, thus giving the impression of neuronal loss with age. In fact, several steps in the processing procedures to prepare tissue for examination (e.g. fixing, dehydration, embedding, sectioning, staining), can introduce artificial changes that may differ among experimental groups and subjects.

Design-based morphometry, also termed stereology, is designed to overcome such biases. Parameters such as regional volume, cell size, synapse and neuron number, and fiber length are all quantifiable using stereology. The basic principle of stereology, which makes it unbiased, is that every part of the region of interest, and every object within this region, has an equal probability of being counted, independent of its size, shape, or orientation. Two concepts have often been confused when comparing the advantages of stereology to assumption-based morphometry: precision vs. unbiasedness (West, 1999). Biasedness due to assumption-based estimation cannot be reduced by increasing the amount of sampling that is increasing precision. However, even with a relatively low level of sampling or precision, the average of unbiased estimates corresponds to the true value (see Fig.1). The concept of unbiasedness is accomplished with the aid of two principles: 1. Systematic-random sampling, and, 2. Unbiased probes:

1. Systematic-random sampling is an effective and accurate sampling scheme that ensures that each cell in the region of interest has an equal probability of being counted. It is therefore necessary that the entire region of interest is available for slicing. The term systematic- random refers to the choice of a random section within an initial interval, and to the systematic collection of every section in a fixed interval up to the end of the region under investigation (see Fig.2A). After visualization of the structure of interest, a uniform grid is randomly superimposed on each section, and the grid points that fall into the region of interest are used for further measurements

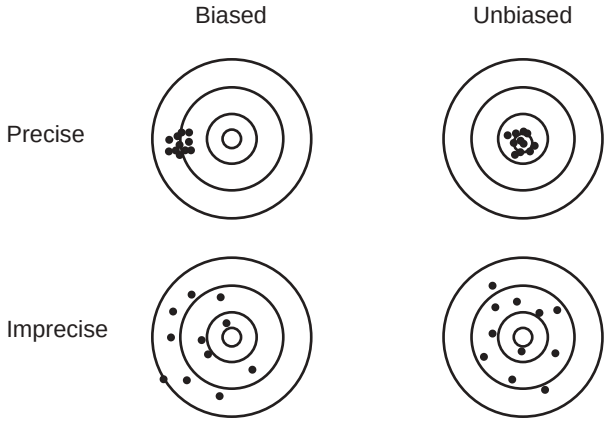

Fig.1: A graphical illustration comparing precision and biasedness in quantitative morphometry. Black dots represent estimates of individual quantifications, whereas the center of the target corresponds to the true value. Increasing the degree of sampling reduces inter-individual differences and increases precision in both biased and unbiased paradigms. Biased methods introduce systematic error regardless of whether effort is made to sample to a high level of precision. In contrast, unbiased estimates converge on the true value in response to increased sampling.

(see Fig.2B,C). In the past, cell counting was performed at ‚representative' locations on only a few selected sections, assuming that the distribution of cells was homogenous. However, cells are usually not distributed uniformly, and procedure artifacts as well as physiological parameters such as age, can add to heterogeneity of cell distribution.

2. Unbiased probes are 3D tools placed virtually within the tissue that allow for unbiased measurements, e.g. independent on the size, shape, and orientation of the structure counted. For the estimation of neurons a sampling probe called the optical disector is used (see Fig. 2D). An optical disector is a virtual stack of focal planes, as viewed along the z-axis of a relatively thick tissue section that allows counting of whole cells (and not profiles). Cells are counted according to simple and unbiased rules, that is, only cells touching the inclusion planes are counted, again ensuring that all cells have the same probability of being sampled. For a complete review of stereological methods see Gundersen and Jensen, (1987); Gundersen et al., (1988); West, (1993).

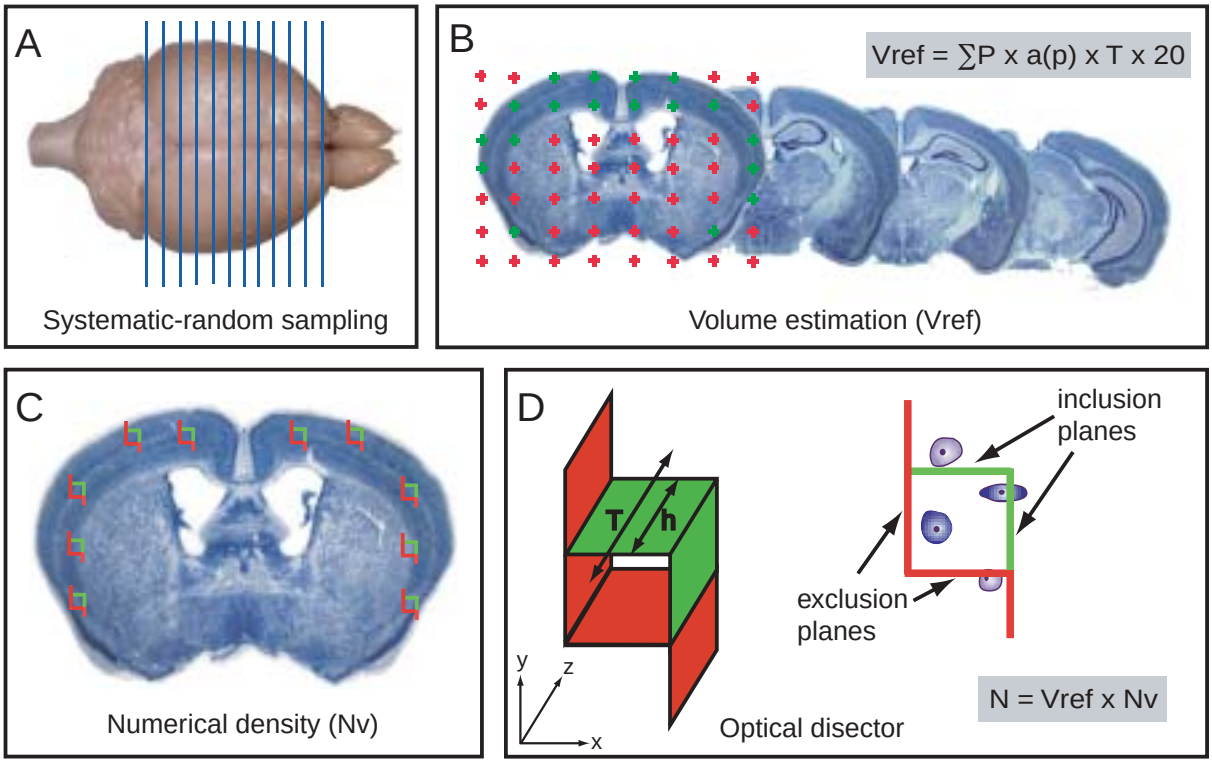

Fig.2: A representative example illustrating the principles of stereology: Estimation of the total neuron number in the murine neocortex. (A), A systematic-random series of every 20th coronal section throughout the entire neocortex is selected, yielding 10-15 sections. (B), The volume of the neocortex is estimated using Cavalieri principles: First a grid, with points separated from each other by a known distance, is superimposed on the section. The area occupied by the points falling inside the neocortex is a good measure for the area occupied by the neocortex. When the thickness of the sections is measured the volume of the neocortex can be calculated: Vref = ∑P x a(p) x T x 20, where ∑P is the number of points counted on all sections, a(p) is the area subtended by each point, T is the mean thickness of the sections and 20 is the sectioning interval. (C), A second grid generates the disector locations, where the numerical density of neurons Nv is counted. (D), On the left an illustration of the optical disector is shown, with a known height h that is virtually placed in the section of thickness T. Using a microscope equipped with a high numerical aperture objective, neurons which fall into the disector boundaries are counted, while focusing along the z-axis. A set of counting rules are applied in order to achieve a reliable estimate for the numerical density of neurons: A dimensionless point on the neuron, such as the nucleolus, is used as the counted object; only neurons which lie within the inclusion planes are counted (dark neurons in the disector), thereby ensuring that every object has an equal probability of being sampled; the disector is placed in the center of the tissue in order to avoid processing artifacts at the section edge. The numerical density can be calculated with the following formula: Nv = Q / (Vdis x # disectors), where Q is the number of neurons counted, and Vdis is the volume of the disector. Now the total number of neurons N can be calculated by multiplying the volume estimate Vref with numerical density Nv: N = Vref x Nv.

1.2 NEUROGENESIS IN THE AGING BRAIN

A central assumption of neuroscience has been that neural stem cells are depleted in the perinatal mammalian brain and that neurogenesis ceases at this time. Several reasons account for the perseverance of this dogma. First, it was inconceivable how new neurons, with their complex architecture, could integrate into the brain without disrupting the existing neuronal network. Second, in contrast to microglia, astrocytes and oligodendrocytes, neurons show no mitotic activity and are generated from progenitors in only two rather discrete regions of the brain. Third, labeling techniques to identify reliably newly generated neurons have only recently been established. Today it is well accepted that neural stem cells persist in the adult brain and support ongoing neurogenesis.

1.2.1 Historical aspects of neurogenesis

In the early sixties Joseph Altman made his original claim of neurogenesis, reporting new neurons in the dentate gyrus (DG) of the olfactory bulb in rats (Altman and Das, 1965). He used the newly introduced 3H-thymidine, which incorporates into the DNA of dividing cells and can be detected with autoradiography. At that time neuronal markers were not available and Altman looked only at the lightmicroscopic level, thus he could not prove unambiguously that the adult-generated cells were in fact neurons. Only two decades later strong support for Altman‘s claims came from studies in rats and macaques by combining 3H-thymidine radiography with electron microscopy (Kaplan and Bell, 1984). However, the general acceptance of neurogenesis in the adult brain came only with the experiments of Fernando Nottebohm and colleagues in songbirds, together with the introduction of new methods for distinguishing neurons from glia and for labeling new cells. This labeling technique takes advantage of the synthetic thymidine analogue BrdU (5-Bromodeoxyuridine) that is incorporated into the DNA during the S-phase of mitosis (Nowakowski et al., 1989). BrdU can be detected immunocytochemically, and when neuron specific markers are used, the existence of neurogenesis can unambiguously be proven.

Using these techniques neurogenesis has been shown to occur in adult mammals, ranging from rodents to primates, in the DG of the hippocampus (Altman and Das, 1965; Kaplan and Bell, 1984; Eriksson et al., 1998; Gould et al., 1998), and the olfactory bulb (Altman and Das, 1966;

Lois and Alvarez-Buylla, 1993; Kornack and Rakic, 2001b). Furthermore, low numbers of new neurons have been reported in additional parts of the hippocampus, in the substantia nigra, and in the cortex (Gould et al., 1999b; Rietze et al., 2000; Zhao et al., 2003), although the latter remains controversial (Kornack and Rakic, 2001a).

New neurons in the olfactory bulb (OB) originate in the anterior part of the subventricular zone (SVZ) (Luskin, 1993; Doetsch and Alvarez-Buylla, 1996), which is the largest germinal layer of the adult brain (see Fig.3). From the SVZ, proliferative cells migrate to the OB via the

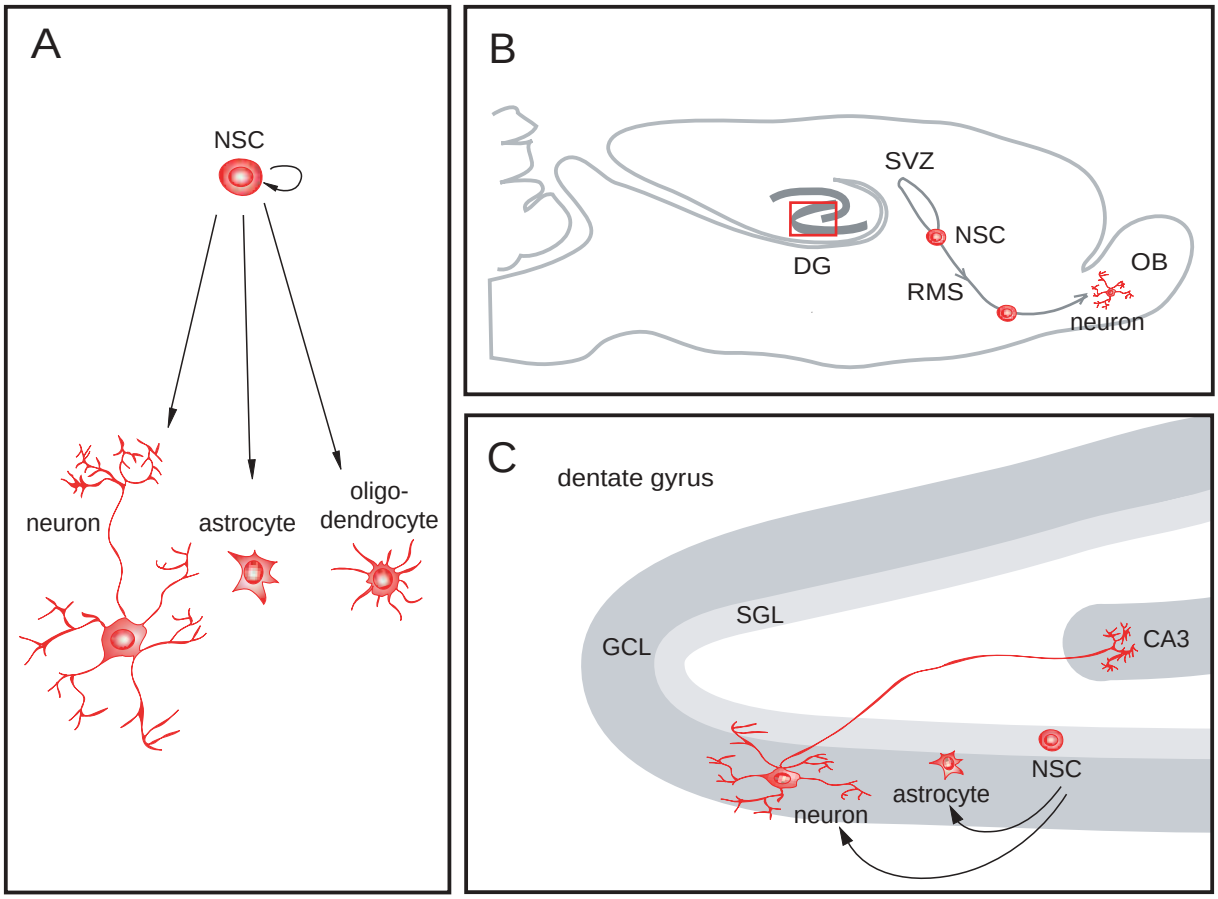

Fig.3: Neurogenesis and neural stem cells (NSC) of the adult mammalian CNS. (A), NSCs are self-renewing, multipotent cells that can generate cells of the neuronal lineage: neurons, astrocytes, and oligodendrocytes. (B), Sagittal representation of the two neurogenic regions of the adult CNS: the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus. The new neurons in the OB are generated from NSCs of the subventricular zone (SVZ). The subventricular zone is a narrow zone of tissue in the wall of the lateral ventricle. The NSCs of the SVZ migrate to the OB via the rostro-migratory stream (RMS), where they differentiate into interneurons of the OB. (C), New neurons and astrocytes in the adult DG are generated from NSCs of the subgranular layer (SGL). During differentiation, neurons extend axons to the CA3 region of the hippocampus.

rostromigratory stream, where they differentiate into interneurons and glia (Lois and Alvarez-Buylla, 1994; Doetsch et al., 1997). The progenitor cells of the hippocampus are produced at the border between the subgranular layer (SGL) and the granular cell layer (GCL) of the DG. They migrate into the GCL, where a portion differentiates into neurons, which then extends axons to the CA3 region (Stanfield and Trice, 1988; van Praag et al., 2002).

1.2.2 Function of neurogenesis

Although the functional significance of adult neurogenesis is still debated, several lines of investigation imply that neurogenesis might be important for learning and memory:

Canaries learn new song elements every year during the breeding season. This acquisition is paralleled by an enlargement of the song control nuclei through the addition of new neurons (Alvarez-Buylla et al., 1992). Interestingly, in zebra finches, which learn one song during adolescence and never change it, large numbers of neurons are added to these nuclei only when they are young and not thereafter. When the neurons of these nuclei are selectively killed the birds' song is markedly degraded, however their song repertoire returns just as neurogenesis starts to replenish the lost neurons (Scharff et al., 2000).

The involvement of hippocampal neurogenesis in spatial learning comes from studies in black-chapped chickadees, which hide seeds in fall to retrieve later. The number of newborn neurons peak when the birds are storing seeds, and the extent of neurogenesis correlates with the flying distance to the hiding place (Barnea and Nottebohm, 1994).

Studies with NCAM knockout mice, in which the number of newly formed SVZ cells that reach the OB is dramatically reduced, show an impaired odor discrimination (Gheusi et al., 2000), whereas a doubling of new olfactory interneurons enhances new odor memory (Rochefort et al., 2002). A recent study has identified the hormone prolactin, whose concentration is increased during pregnancy, to trigger rodent neurogenesis in the OB, but not in the DG (Shingo et al., 2003). As mice deficient for the prolactin receptor tend to ignore their young (Lucas et al., 1998), it could be speculated that increased neurogenesis improves olfactory discrimination and contributes to maternal behavior (Shingo et al., 2003).

Mice living in an enriched environment show increased survival rates of new neurons in the DG, which are paralleled by an improvement on a hippocampal-dependent learning task (Barnea and Nottebohm, 1994; Kempermann et al., 1997; Nilsson et al., 1999; Kempermann et al., 2002). A similar survival-promoting effect has been reported in rats after performing an associative learning task (Gould et al., 1999a). Moreover, decreasing the number of new granule neurons with a cytostatic agent is correlated with impairment on such a task (Shors et al., 2001).

1.2.3 Modulation of neurogenesis

Neurogenesis can be stimulated by several conditions, indicating an important role for brain function and offering a possible key for understanding or even treating neurodegenerative diseases associated with neuron loss. Growth factors that have been shown to influence neurogenesis are insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). IGF-1 infusion restores hippocampal neurogenesis in aged rats (Lichtenwalner et al., 2001) and increases the fraction of newborn neurons in IGF-1 deficient mice (Aberg et al., 2000). Moreover, mice overexpressing IGF-1 have increased volume and numbers of neurons in the GCL of the DG (O'Kusky et al., 2000). Infusions of both EGF and FGF-2 increase the number of newborn neurons in the SVZ (Kuhn et al., 1997). In vitro cultured stem cells proliferate and express neuronal and glial markers when grown in defined media containing EGF or FGF-2 (Reynolds and Weiss, 1992; Gage et al., 1995). Hormones have been identified that control neurogenesis: Maternal hormones such as estrogen and prolactin enhance the production of new neurons (Tanapat et al., 1999; Shingo et al., 2003). Furthermore a decrease of neurogenesis after ovarectomy is reversed by estrogen replacement (Tanapat et al., 1999).

The modulation of neurogenesis in mice living in an enriched environment has been attributed to two factors, locomotor activity and learning. Neurogenesis is boosted in both animals trained on hippocampal-dependent tasks (Gould et al., 1999a) and in animals that have access to a running wheel thus enhancing their physical activity (van Praag et al., 1999). In contrast, stressful experiences decrease the number of newborn hippocampal neurons (Gould et al., 1997; Tanapat et al., 2001).

Aging has been shown to decrease the proliferative rate of neural stem cells in rats (Seki and Arai, 1995; Kuhn et al., 1996). Given that newly produced neurons are suggested to play an important role in learning and memory (van Praag et al., 1999; Ambrogini et al., 2000; Shors et al., 2001), age-related restrictions in neuronal turnover could represent a correlate for the cognitive decline with aging (Cameron and McKay, 1999). The decrease in neurogenesis may be explained by a reduction in trophic factors and glucocorticoids, which are implicated in hippocampal aging processes (Landfield et al., 1978; Sapolsky et al., 1985; Stenvers et al., 1996; Bhatnagar et al., 1997; Bizon et al., 2001). Indeed, reducing glucocorticoid levels by adrenalectomy restores the rate of cell proliferation in aged rats (Cameron and McKay, 1999). These results suggest that the population size of neuronal precursors does not diminish with age, but that neurogenesis is slowed by high corticosteroid levels. Alternatively, a limited proliferation and differentiation of pools of neuronal progenitors in the DG could lead to the age-related decrease in neurogenesis as the pool of neuronal progenitors declines (Seaberg and van der Kooy, 2002).

1.3 ALZHEIMER'S DISEASE (AD)

The increase in life expectancy during the last fifty years has enabled many human beings to reach an age at which degenerative diseases of the brain become common. As late as the middle of this century, dementia was assumed to be a natural accompaniment of old age. In recent years, it has become clear that the brains of more than half of the people with dementia show all the characteristic signs of Alzheimer's disease (AD) when examined at autopsy.

1.3.1 Epidemiological and clinical aspects

Worldwide epidemiological studies suggest that between 5 to 15% of people in their 70s, and even 15 to 40% in their 80s, suffer from AD (McKhann et al., 1984; Evans et al., 1989; Lautenschlager et al., 1996). Recent demographic changes reveal significant shifts in life expectancy, and thus indicate that an increasing portion of people will be at risk for AD in the

future (Olshansky, 1993). This drastic prognosis explains the growing interest in understanding the risk factors and mechanisms leading to AD and in developing AD treatments.

Clinically, AD starts with occasional episodic memory deficits, such as difficulties in recalling recent events of daily life. This syndrome is often referred to as mild cognitive impairment (Morris et al., 1996; Petersen et al., 1999). In the course of the disease problems in executive functions are encountered, that is, orientation in time and place and execution of complex tasks are impaired. Occasionally, patients suffer from personality disturbances including anxiety, aggressiveness, depression or hallucinations (Mayeux and Sano, 1999). Over several years AD patients deteriorate into a marked dementia, with full disorientation, profound memory impairment, and global cognitive deficits. Finally, patients become immobile and die of minor respiratory difficulties such as aspiration or pneumonia (Forstl and Kurz, 1999).

1.3.2 Histopathological characteristics

The classic neuropathological hallmarks of AD include neuronal loss in brain regions critical for learning and memory (Regeur et al., 1994; West et al., 1994; Gomez-Isla et al., 1996), parenchymal and cerebrovascular β-amyloid deposition and intracellular neurofibrillary tangles (NFT) (Selkoe, 2001). NFT are composed of microtubule-associated protein tau in a hyperphosphorylated state (Goedert et al., 1988). Tau protein promotes polymerization of tubulin monomers into microtubules, which are components of the intracellular transport system (Mandelkow and Mandelkow, 1995). NFT destabilize microtubules and hamper axonal transport leading to inappropriate protein metabolism and synaptic malfunction. A progressive decline in these functions may significantly contribute to the degeneration of the affected neurons (Goedert, 1997; Lewis et al., 2001). Parenchymal amyloid plaques are extracellular lesions predominantly composed of the fibrillary 40-42 amino acid long β-amyloid peptides (Aβ), with the longer forms deposited earlier and representing the main component of plaques (Roher et al., 1986; Iwatsubo et al., 1994). Aβ is deposited either as amorphous granular aggregates (diffuse plaques), or as dense-core structures, which are associated with dystrophic neurites, reactive astrocytes and activated microglia (Braak et al., 1999). Aβ is an internal degradation product of the type I transmembrane amyloid precursor protein (APP) (Kang et al., 1987).

1.3.3 Genetics of AD

Genetically, AD can be considered as complex and heterogeneous (Tanzi, 1999): Mutations and polymorphisms in multiple genes are involved together with nongenetic, environmental factors. The complete etiological picture of AD remains unresolved, but the inheritance of predisposing genetic factors appears to play an important role. In fact, after age, family history is the second greatest risk factor for AD (Selkoe, 1999). Sporadic or late-onset AD is responsible for the largest proportion of cases, while early-onset or familial AD (FAD) account for less than 5% of all cases (Finch and Tanzi, 1997). FAD can be attributed to autosomal dominant mutations in at least three different genes that encode APP or the presenilins (PS1 and PS2). In 1991 the first genetic linkages to AD were identified as missense mutations in the APP gene located on Chromosome 21 (Chartier-Harlin et al., 1991; Goate et al., 1991). Since then, 14 additional mutations have been discovered in APP, all of which lie within or close to the domain encoding

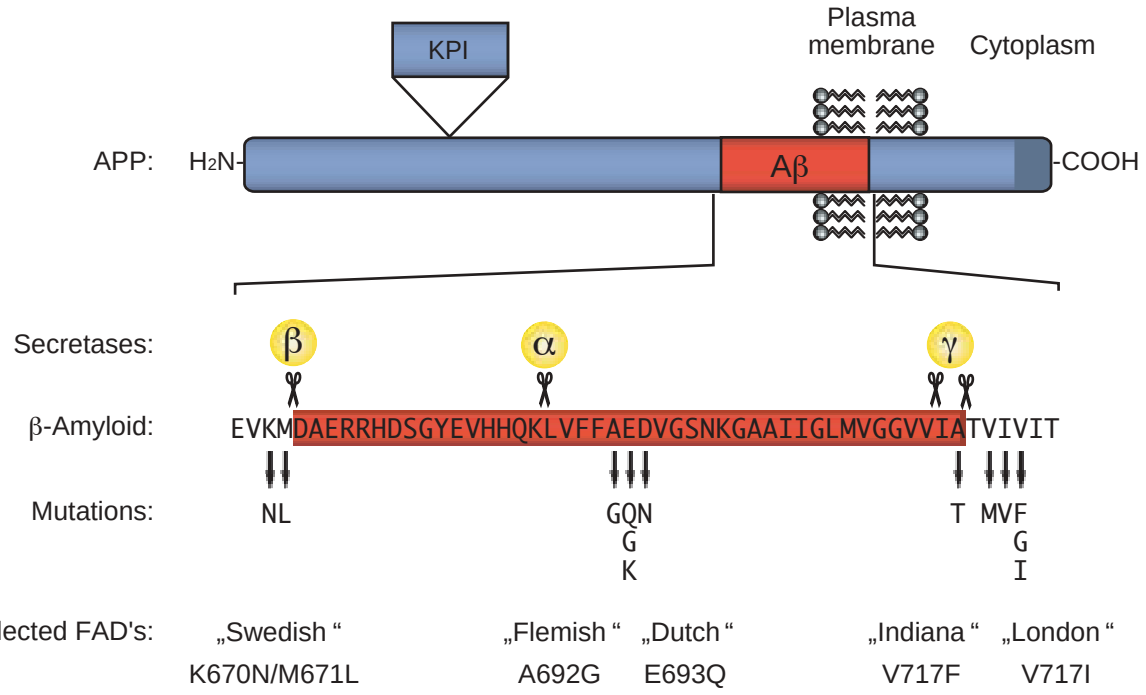

Fig.4: Schematic representation of the β-amyloid precursor protein (APP, in blue), the β-amyloid (Aβ, in red), and missense mutations located within APP. APP is a type I transmembrane protein, which is expressed in three major splice variants of 695, 751, and 770 residues. The two longer forms contain a serine protease inhibitor domain of the Kunitz type (KPI). APP is proteolytically cleaved by sequential actions of α-, β- and γ-secretases (yellow). Mutations on APP are associated with early-onset familial AD (FAD), and lie within or close to the domain encoding the Aβ gene. Mutations near the β- and γ-secretase cleavage sites, either increase total Aβ concentration or levels of the more fibrillogenic $A\beta_{42}$, respectively, whereas mutations inthe middle part of Aβ invariably lead to enhanced cerebrovascular pathology.

the Aβ gene (see Fig.4) (reviewed in Ghiso and Frangione, (2002)). These mutations account for only 1% of FAD cases, while the majority of the familial forms of AD are related to missense mutations in the two presenilin molecules (Hardy, 1997). The presenilins, located on chromosome 14 and 1 (Rogaev et al., 1995; Sherrington et al., 1995) have been implicated in the proteolytic processing of APP (Esler and Wolfe, 2001). According to the latest data a total of 138 mutations have been identified in the presenilins genes, most of them located on presenilin1 (see AD mutations database: http://molgen-uia.ac.be/ADMutations).

Besides these highly penetrant mutations leading to FAD, inheritance of the apolipoprotein E4 allele (ApoE4) is the major susceptibility factor for the development of sporadic AD with lower penetrance but higher prevalence (Saunders et al., 1993; Strittmatter et al., 1993). ApoE is involved in the mobilization and redistribution of cholesterol during normal growth and following injury (Mahley and Huang, 1999). Whereas inheritance of ApoE4 increases the risk of developing AD by up to 10-fold (Mahley and Rall, 2000), the ApoE2 isoform appears to confer some protection from AD (Corder et al., 1994). ApoE4 promotes the deposition of Aβ in the brains of carriers and in transgenic mice (Poirier, 2000), and decreases the age of AD onset in a dose-dependent manner (Meyer et al., 1998).

Recent estimates suggest that the four AD genes aforementioned account for less than 30% of the variance in this disease and predict the existence of additional AD genes. In fact, complete genome screenings point to candidate regions on chromosome 12, 10, and 9 (Pericak-Vance et al., 1997; Kehoe et al., 1999). The putative AD locus on chromosome 12 is associated with the genes that encode α-2 macroglobulin and its receptor, whereas the candidate gene on chromosome 10 codes for the insulin-degrading-enzyme (IDE) (Bertram et al., 2000; Farris et al., 2003). All these genes have been implicated in the degradation of Aβ.

1.3.4 APP and its proteolytic cleavage

The expression of APP involves complex differential splicing. The primary RNA transcripts generate mRNAs coding for three major isoforms of 695, 751, and 770 residues (Selkoe, 2001). The two longer isoforms contain a motif, which is homologous to the Kunitz-type of serine protease inhibitors (KPI). The heterogeneity of APP arises as well by a variety of posttranslational modifications, including glycosylation, sulfation, and phosphorylation.

At present the exact function of APP remains to be clarified. The high degree of evolutionary conservation and its widespread tissue distribution suggests important functions (Johnstone et al., 1991). Based on cell culture studies, APP and its secreted forms have been implicated in cell proliferation, neuroprotection, and neuron viability (Saitoh et al., 1989; Mattson et al., 1993; Perez et al., 1997). These functions have yet to be clearly confirmed in vivo. Deletion of APP in mice does not lead to vital consequences, maybe due to the compensatory expression of proteins homologous to APP (Wasco et al., 1992; Slunt et al., 1994).

A characteristic feature of APP is its proteolytic cleavage (see Fig.5). APP is processed by three types of proteases, designated α-, β- and γ-secretases. The differential action of these secretases leads either to the nonamyloidogenic, or amyloidogenic pathway, that are distinguished as follows.

Most APP molecules undergo non-amyloidogenic processing. The α-secretase cleaves APP within the exoplasmatic portion of Aβ, primarily at residue 16, releasing a soluble ectodomain of APP (APPs-α) (Esch et al., 1990; Sisodia et al., 1990). Three candidate α-secretases have been suggested, namely ADAM (a disintegrin and metalloprotease) 9, 10, and 17, all of them acting as zinc-dependent metalloproteases (Asai et al., 2003). The membrane retained C-terminal fragment (C83) is then cleaved by the γ-secretase, generating the p3 peptide

The amyloidogenic pathway generates Aβ by the sequential action of two aspartyl proteases, the β- and γ-secretases (Selkoe, 2001). As 12 to 14 residues of Aβ extend into the membrane, it was originally unclear how cleavage could occur. The amyloidogenic pathway was thought to be coupled to membrane disruption and Aβ was described as being a pathological metabolite of APP. This concept was disproved a decade ago, when Aβ was shown to be constitutively produced by mammalian cells throughout life and to occur normally in plasma and cerebrospinal fluid (Haass et al., 1992; Seubert et al., 1992).

β-secretase cleavage generates a soluble portion (APPs-β) and the membrane-bound fragment C99, starting at the N-terminus of Aβ. In contrast to the other two secretases, β-secretase has been clearly identified and its crystal structure solved (Vassar et al., 1999; Hong et al., 2000). β-secretase, or BACE1 is a single-transmembrane protein that is highly expressed in the brain. It is situated on chromosome 11 and contains two active site aspartate residues (Esler and Wolfe, 2001).

The molecular events involved in γ-secretase cleavage that release Aβ are complex and are still debated. This is due to its unusual ability to cut in the middle of the transmembrane region of APP. Moreover, γ-secretase appears to be a multimeric complex, therefore complicating its

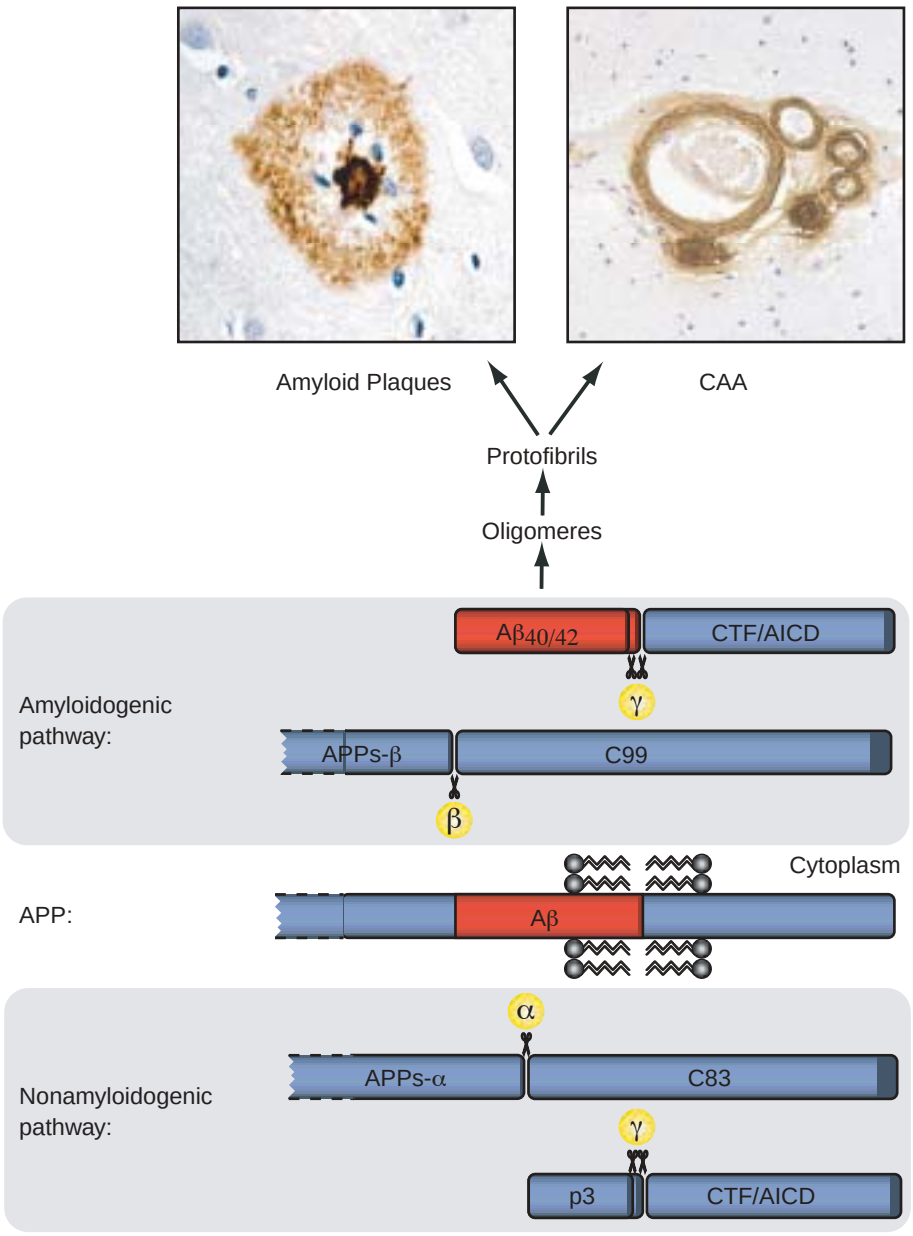

Fig.5: Pathways of APP processing by α-, β- and γ-secretases. APP can be processed along the nonamyloidogenic or the amyloidogenic pathway. In the nonamyloidogenic pathway α-secretase cleaves in the middle of the Aβ region to release a large soluble APP fragment, APPs-α. The 83 amino acid long fragment C83 is metabolized by the γ-secretase to produce p3 and the C-terminal fragment (CTF, also termed APP intracellular domain, or AICD). In the amyloidogenic pathway, β-secretase cleavage produces the 99 amino acid long peptide C99 and the soluble APPs-β fragment. C99 is then metabolized to produce the CTF/AICD peptide, and the neurotoxic $A\beta_{40}$ and $A\beta_{42}$ ($A\beta_{40/42}$), by the action of γ-secretase. Whereas CTF/AICD may be targeted to the nucleus to act as a transcription factor, $A\beta_{40/42}$ is secreted into the extracellular space, where it can be deposited in amyloid plaques or in the cerebral vasculature (CAA).

identification. It is suggested that this cleavage involves at least four components: the presenilins, nicastrin, aph-1, and pen-2 (De Strooper et al., 1998; Yu et al., 2000b; Francis et al., 2002). The presenilins have been suggested to contain the active site of the γ-secretase. In fact, mutations of either of the two critical aspartate residues in the presenilins prevent their heterodimer formation and block γ-secretase cleavage of APP (Wolfe et al., 1999; Yu et al., 2000a). In addition, FAD autosomal dominant mutations in the presenilins enhance $A\beta_{42}$ generation (Selkoe, 2001), also supporting the hypothesis that they are a critical component of γ-secretase.

1.3.5 Mouse models of AD

Genetically-engineered mouse models present an important system for in vivo studies of the pathophysiology of neurodegenerative diseases such as AD. The use of transgenic and knock-out techniques allows for the testing of hypotheses regarding the function, or dysfunction, of specific proteins and the impact of genetic alterations. However, considerable effort must be exerted to demonstrate that the observed phenomena are not due to normal aging or idiosyncratic developmental processes. The standard way to do this in the field of transgenic mouse models is to compare the phenotype of mice expressing a mutated transgene with that of nontransgenic littermates. In order to detect the mechanisms underlying the pathogenesis in transgenic mouse models for AD, it is important to understand normal age-associated changes in the brain of wildtype mice. The development of stereological techniques provides an accurate method to quantify such changes. B6 mice are one of the most frequently used inbred mouse strains in neuroscience, including use as a background strain for transgenic studies (Ingram and Jucker, 1999). Considerable baseline behavioral and morphometrical parameters have been collected in aging B6 mice, including counts of neurons, microglia, astrocytes and synapses (Calhoun et al., 1998a; Long et al., 1998; Mouton et al., 2002). These results provide a solid basis for comparisons with other distinct mouse strains, and with genetically-engineered mice on a B6 background.

In an effort to clarify the role of genetic factors in vivo, transgenic mice have been generated that express genes involved in AD: mutant and wildtype APP, PS1 and PS2, ApoE, and Tau. Although the early results were disappointing because the mice did not show the neuropathological hallmarks of AD, more recent work has yielded multiple lines of transgenic mice that show

Aβ-deposits, tangle formation, and cognitive impairments (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Lamb et al., 1999; Lewis et al., 2000). However, no model develops all aspects of AD neuropathology, which might be desirable to test effectiveness and side effects of therapeutic agents against AD. Nevertheless, transgenic mice have proven exceptional tools to elucidate the distinct roles of genes involved in the pathogenesis of AD.

The accepted central role of APP in the process of AD is double: as the direct precursor of Aβ, and because mutations in APP that cause FAD and all increase production of Aβ (Selkoe, 2001). Therefore, much effort has been devoted to generate transgenic mice that express mutant human APP (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Van Dorpe et al., 2000; Chishti et al., 2001). These mice exhibit AD-like pathologies such as neuron loss, dystrophic neurites, glial activation, and learning and memory deficits (Janus and Westaway, 2001). Most importantly, the overexpression of mutated APP leads to an age-dependent extracellular deposition of Aβ. The nature of this deposition is heterogeneous, depending on the transgenic mouse line. While some mouse lines have predominantly diffuse Aβ deposition (Games et al., 1995), other lines exhibit mostly compact plaques (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997). Furthermore, in some APP transgenic mouse lines deposition of Aβ has been reported in the cerebral vasculature (Calhoun et al., 1999; Van Dorpe et al., 2000). These different phenotypes may be explained by the APP mutation, the gene-promotor, the copy number and integration site, or the mouse genetic background.

One such transgenic mouse model is the APP23 mouse. APP23 mice overexpress human APP_{751}, bearing the Swedish double mutation K670N and M671L, under the control of a neuron-specific Thy-1 promoter (Sturchler-Pierrat et al., 1997). APP23 mice have initially been produced on a B6D2 background but have been backcrossed to B6 for more than 10 generations. Amyloid deposition in these mice starts at 6 months of age. Amyloid-associated neurodegeneration has been described in detail in previous studies (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998b; Phinney et al., 1999; Stalder et al., 1999; Boncristiano et al., 2002).

1.3.6 Therapeutic approaches

At present, there is no effective way to prevent or cure AD. Most available agents belong to the class of the acetycholinesterase inhibitors that are aimed at counterbalancing deficits in the neurotransmitter acetylcholine observed in AD. These drugs induce, at best, only short-term improvements in cognitive decline, but do not stop its progression (Grutzendler and Morris, 2001). In the past years, the ‚amyloid cascade hypothesis', which predicts that Aβ has a critical role in all AD cases, has become the dominant theory of AD pathology (Hardy and Allsop, 1991). The proof whether Aβ causes AD or not should ultimately come from clinical trials with Aβ lowering agents. This could be achieved by decreasing Aβ production, increasing Aβ clearance, or interfering with Aβ aggregation and precipitation into fibrils and plaques.

Epidemiological studies suggest that cholesterol-lowering and anti-inflammatory drugs provide some degree of protection from AD. In fact, high blood cholesterol levels have been correlated with a higher risk of developing AD (Kivipelto et al., 2001). Treatments with statins, which are inhibitors of the cholesterol-synthesizing enzyme HMG-CoA-reductase, protect from dementia (Jick et al., 2000). A molecular explanation might be that high cholesterol favors processing of APP through the amyloidogenic beta-secretase pathway in vitro and in vivo (Simons et al., 1998; Fassbender et al., 2001). Studies have also shown reduced incidence and slower progression of AD in patients treated with non-steroidal anti-inflammatory drugs (NSAIDs) (in t' Veld et al., 2001). The anti-inflammatory drug ibuprofen reduces plaque deposition and associated pathology in transgenic mice (Lim et al., 2000), perhaps by lowering the production of the highly amyloidogenic Aβ (Weggen et al., 2001).

The enzymatic activities of the β- and γ-secretase cleave APP releasing Aβ. Therefore, development of protease inhibitors could reduce the production of Aβ. Inhibitors of γ-secretase have been shown to reduce Aβ levels in the brain of a mouse model of AD (Dovey et al., 2001). A major concern is the involvement of the γ-secretase in the cleavage of other membrane proteins such as Notch (Strooper and Annaert, 2001). In fact, Notch signaling is important for a variety of cell fate decisions during embryogenesis and also in adult tissues (Milner and Bigas, 1999; Doerfler et al., 2001; Sisodia and St George-Hyslop, 2002). Knockout of PS1 is lethal, and is likely due to the role of γ-secretase in processing Notch (Sisodia and St George-Hyslop, 2002).

However, lowering steady-state levels of Aβ by 30% with only partial inhibition of γ-secretase may prove therapeutically beneficial and leave a sufficient reserve of notch signaling (Selkoe and Schenk, 2003). β-secretase has received much attention in drug development, since its crystal structure was solved (Hong et al., 2000). Furthermore, β-secretase knockout mice are apparently normal showing no obvious anatomical or physiological abnormalities (Luo et al., 2001; Roberds et al., 2001). One major challenge for the generation of specific inhibitors is the unusually large active site of this secretase (Hong et al., 2000). This requires the identification of small organic molecules that contact the active site at several positions.

An alternative pathway to reduce Aβ burden in the brain would be to interfere with Aβ catabolism. Recently, several Aβ-degrading enzymes have been proposed including the metalloproteases insulin-degrading enzyme (IDE) and neprilysin (Qiu et al., 1998; Iwata et al., 2000). IDE catabolizes Aβ in neuronal and microglial cultures (Vekrellis et al., 2000) and IDE knockout mice exhibit increased levels of Aβ (Farris et al., 2003).

In 1999, anti- Aβ vaccination emerged as an unexpected potential treatment for AD (Schenk et al., 1999). The APP transgenic mouse model used, showed reduced plaque pathology following active immunization with synthetic Aβ. Interestingly, vaccination not only prevented amyloid deposition in young mice but also cleared plaques and reduced associated glial and neuronal cytopathology in older animals. Moreover, in other mouse models of AD, immunization with Aβ was found to protect mice against learning and memory deficits (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002). Removal of amyloid deposition was successful in different strategies (see Fig.6): Vaccination with the Aβ peptide (active immunization) (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000), or direct anti-Aβ antibody injections (passive immunization) (Bard et al., 2000; DeMattos et al., 2001).

Possible mechanisms responsible for the clearance of Aβ involve:

1. Antibody-induced disaggregation of amyloid fibrils. Antibodies directed to the amino-terminus of Aβ were shown to prevent its fibrillar aggregation and to disrupt Aβ fibers in vitro (Solomon et al., 1996; Solomon et al., 1997).

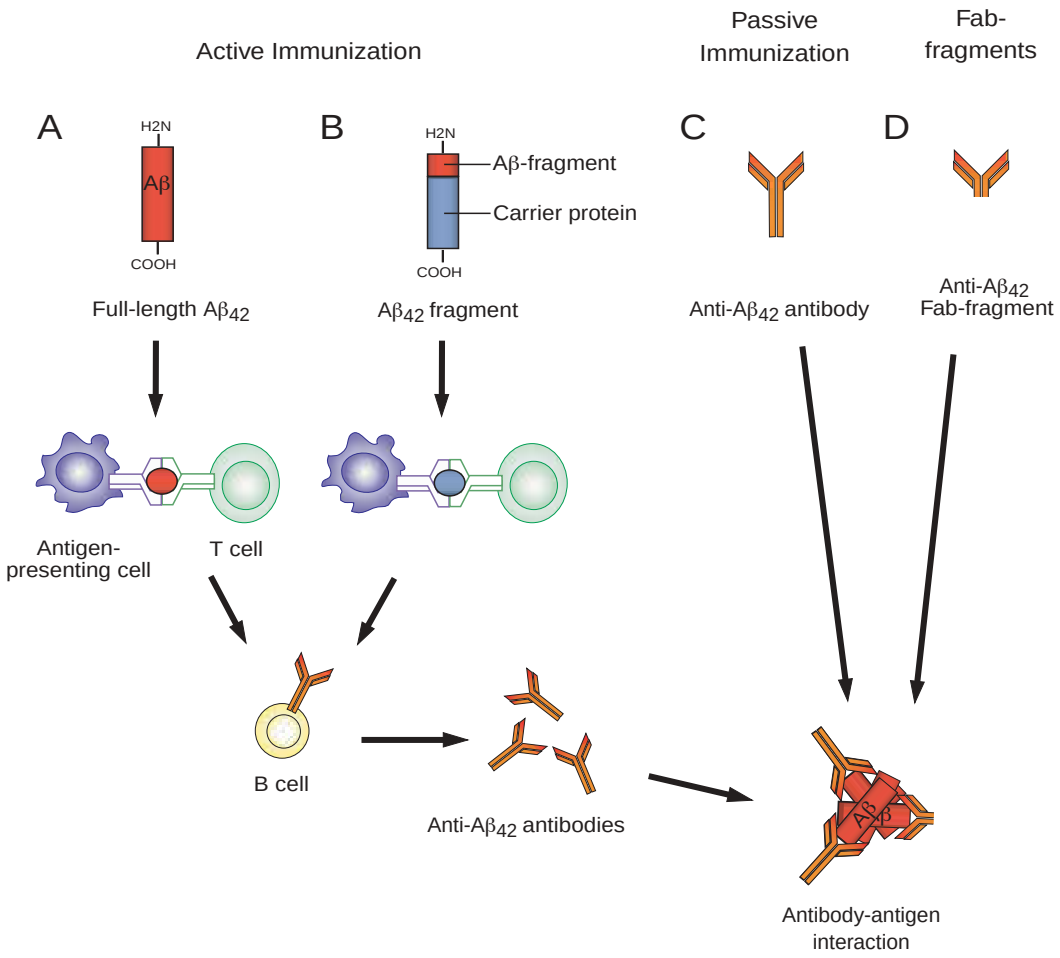

Fig.6: Illustration of four different antibody strategies targeted at clearing Aβ. (A) The first approach involves administration of full-length Aβ. The peptide is internalized by antigen-presenting cells, and fragments are presented to T cells. Thereafter, B cells that recognize epitopes on $A\beta_{42}$ are involved and proliferate. These B cells produce antibodies against Aβ. (B) In a second approach N-terminal Aβ-fragments that contain a predicted B cell-activating domain but lack the T-cell-activating epitopes are coupled to an unrelated carrier protein. In this so-called 'immunoconjugate strategy' the T-cell epitopes are provided by the carrier protein, thus avoiding the potential side effects associated with mounting a T cell response directly against Aβ. (C) Another way for immunization is to administer Aβ-antibodies directly (passive immunization). This induces a humoral response, without triggering T-cells at all. (D) In the fourth approach anti-Aβ antibodies are used that lack the Fc region (Fab-fragments). This circumvents Fc-related phagozytosis and is thought to directly disrupt amyloid deposits.

2. Fc-receptor mediated phagocytosis of Aβ by microglial cells (Bard et al., 2000). In this model antibodies cross the blood-brain-barrier and target aggregated Aβ deposits where they form immune complexes. The Fc-region of the anti-Aβ antibodies binds to the Fc-receptors on microglia and induces phagocytosis of the Aβ peptide.

3. Drainage of soluble Aβ into the plasma (DeMattos et al., 2001). This third mechanism of action suggests that anti-Aβ antibodies act as a peripheral sink by enhancing the efflux of Aβ from the brain into the plasma.

Regardless of the mechanism, antibodies to Aβ are efficacious in reducing plaque burden in transgenic mouse models. Based on these results, Elan Corporation and its collaborating company, Wyeth-Ayerst, moved into clinical trials with the active Aβ-vaccination approach. Toxicological testing in several animal species, and Phase I clinical trials in 104 AD cases suggested that the vaccination with aggregated $A\beta_{42}$ was safe (Senior, 2002). Therefore, in June 2001, a Phase II trial was begun with 360 early to moderate stage Alzheimer's patients in the United States and Europe. However, the trial was suspended in January 2002, when 6% of the patients developed clinical signs of meningoencephalitis, a potentially deadly inflammation of the brain (Orgogozo et al., 2003). The mechanism of this inflammation is still poorly understood, but the appearance of the inflammatory reaction before anti-Aβ antibody production in some patients indicates the involvement of a T-cell-mediated immune response to Aβ (Weiner and Selkoe, 2002). In addition, the first post-mortem examination of human neuropathology after immunization, showed a widespread T-lymphocyte meningoencaphalitis (Nicoll et al., 2003). In line with this observation, the C-terminus of $A\beta_{42}$ was shown to contain a T-cell-activating domain (Monsonego et al., 2001).

Strategies circumventing such side effects include:

1. Administration of N-terminal Aβ-fragments that do not contain the T-cell-activating epitope against Aβ can still reduce both aggregation and cytotoxicity of Aβ in vitro (McLaurin et al., 2002). The coupling of Aβ-fragment to a carrier protein that provides helper T-cell epitopes (immunoconjugate approach) also results in decreased AD-like pathology in transgenic mice (Sigurdsson et al., 2001).

2. Use of anti- Aβ antibodies lacking the Fc region of the antibody (Fab-fragments) was sufficient to reduce amyloid burden in transgenic mice (Bacskai et al., 2002). This suggests that in addition to Fc-related phagocytosis, a direct disruption of plaques is involved.

3. Passive immunization with humanized anti-Aβ antibodies that trigger a humoral response alone, without involving T-cells (Bard et al., 2000; DeMattos et al., 2001; Dodart et al., 2002), was shown to reverse memory impairments, with and without reducing Aβ burden.

Despite the setback in the immunization trial due to the serious side effects encountered, autopsy evaluation and cognitive assessment of patients involved in the study suggest that immunization might work (Hock et al., 2003; Nicoll et al., 2003). The patients included in the small Zürich cohort developed high titers of anti-Aβ antibodies, which selectively recognize fibrillar Aβ (Hock et al., 2002). Subsequent testing revealed diminished cognitive decline and slowed disease progression in patients that generated anti-Aβ antibody titers (Hock et al., 2003). The autopsy case revealed a strong reduction of plaque deposition in the temporal lobe, a region that is usually heavily affected in AD patients (Nicoll et al., 2003). This area also showed low levels of dystrophic neurites and astrocytic clusters. Furthermore, the low Aβ-immunoreactivity in these regions was associated with activated microglia, which might be involved in the clearance process. In contrast, other lesions associated with AD pathology, such as neurofibrillary tangles and Aβ deposits in the vasculature where not reduced in these regions. Most promising, patients of the Zürich cohort, which generated anti-Aβ antibodies titers revealed diminished cognitive decline as well as slowed disease progression (Hock et al., 2003)

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2. IMPACT OF AGE AND CALORIC RESTRICTION ON NEUROGENESIS IN THE DENTATE GYRUS OF C57L/B6 MICE

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Summary

Age-related changes in neurogenesis and its modulation by caloric restriction 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. Caloric restriction 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 caloric restriction does not counteract this age-related decline in neurogenesis but promotes survival of hilar glial cells.

Key words: mouse brain, aging, neurogenesis, glia, caloric restriction, hippocampus

2.1 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 [2,10,13,19,22]. Neurogenesis has been described in the subgranular layer of the dentate gyrus [2,18] and the subventricular zone of the lateral ventricle [3,21,27]. 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 [14,30,43]. 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 [4,37,42]. Because a decline of neurogenesis in the dentate gyrus has been reported with aging [12,22], a relationship between the age-related decline in neurogenesis and age-dependent cognitive impairments may be suggested [8].

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 [31,38]. 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 [24,25].

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 [16,17]. Moreover, we have previously assessed age-related changes in granule cell number in the dentate gyrus of this mouse strain [7] and thus have provided a basis for the interpretation of age-related changes in neurogenesis in the dentate gyrus of this mouse strain.

2.2 Materials and Methods

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: 24.4g, 31.5g, 32.6g, and 32.0g (SEM ±0.7 - ±1.3g). 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 (CR, n=9) were used. Their respective mean body weights were: AL fed, 31.3±0.7g; CR, 23.8±0.5g. 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 [41]. All mice had been housed in standard cages in a colony maintained by the National Institute on Aging at Harlan Sprague-Dawley (Indianapolis, IN).

BrdU labeling and cellular phenotyping

For labeling of newly generated cells mice were given daily single injections of bromodeoxyuridine (BrdU; 50μg/g body weight i.p., Sigma) for 5 consecutive days. Half of the mice of each age group were killed 2 hours 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 four 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 [22]. Briefly, sections were incubated in 50% formamide in 2x SSC for 2 hrs at 65°C, followed by 10 min in 2x SSC, 30 min in 2N HCl at 37°C and 10 min in 0.1M borate buffer. Sections were then incubated in 0.08% H2O2, 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 S100b (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 100 M (Zeiss).

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 [44]. Quantifications were performed on systematic random series of every 6th 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 the standard error of the mean (SEM). Statistical significance was accepted as $p<0.05$.

2.3 Results

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 hours and 4 weeks after the last BrdU injection. Two hours post-BrdU, cell proliferation was predominantly conferred to the subgranular layer (Fig. 1A,C,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,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 hour-post-BrdU and the 4 week-post-BrdU group revealed only few BrdU-positive cells (Fig.1). Their distribution appeared random and an age-related decrease was also apparent.

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 hour- and 4 week-post-BrdU group. 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 month-old 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 hour-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.

To phenotype the newly generated cells, immunofluorescence triplelabeling for BrdU, the neuronal marker NeuN, and the astrocytic marker S100b 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 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/S100b-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 S100b (Table 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 hour-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. 4B vs. 4D). 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 weeks post-BrdU group ($p=0.001$) but not in the 2 hours 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%, 4 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.

2.4 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 hours post-BrdU, whereas a 4 weeks survival period was used to examine cell survival and differentiation.

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 [20] and rats [8,22,26,36]. The reduction of neurogenesis with aging was mainly caused by an age-related decrease in progenitor cell proliferation, whereas cell survival was not changed 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 [8,20,22,26]. Thus, the current findings would suggest greater functional significance of neurogenesis in younger mice with less impact on those approaching their median lifespan.

The decrease in neurogenesis with aging has been explained by an age-related increase in stress-induced glucocorticoids [23,34]. Indeed, adrenalectomized rats show a markedly higher proliferation of neural precursors and no age-related decrease in neurogenesis [8]. Furthermore, physical activity, which has been shown to increase neurogenesis [42], declines in an age-dependent manner in rodents, including C57BL/6 mice [11,15]. Moreover, a decline in the pool of neuronal progenitors [35] or a lengthening of the cell cycle of progenitor cells with aging [40] has been reported. Unfortunately, none of the studies examined whether the decrease in corticosteroids, physical activity, and neural precursor cell pool and/or cell cycle length 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 [8]. 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 [7,16]. 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 [16].

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 [1,39]. Interestingly, in the present study we found a significant age-related decrease in the proliferation of such hilar astrocytes. Similar qualitative assessment in rats revealed also modest age-related decrease in astrocyte proliferation [22,26]. 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 [28,33].

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 [9,29,31], 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 [24,25]. 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 [42]. 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 [24] or did not phenotype BrdU-positive cells in AL fed mice [25] 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 [31,32]. 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 [5,6].

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.

2.5 Figure Captions

Figure 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 hours (A,C) and 4 weeks (B,D) 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 weeks 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,D) compared to 2 month-old mice (A,B). Scale bars are 50μm (A-D) and 10μm (E,F).

Figure 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 hours 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 month-old and 24 month-old mice.

Figure 3: Cellular phenotyping of BrdU-positive cells. BrdU immunofluorescence (red) was combined with the neuronal marker NeuN (green) and the astrocytic marker S100b (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 S100b 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 S100b. Scale bar is 20μm.

Figure 4: Proliferation and survival of BrdU-labeled cells in the dentate gyrus of ad libitum fed (AL) and caloric restricted (CR) mice. Eleven month-old mice were killed 2 hours (A,C) or 4 weeks (B,D) after the last BrdU-injection. No qualitative difference was found when number of newborn cells in the GCL of CR mice (C,D) were compared to AL fed mice (A,B). In contrast,

the survival of BrdU-positive cells in the hilus was increased in the CR mice (arrows in D) when compared to AL fed mice. Scale bar is 50μm

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

Table 1. Statistical parameters of two-way ANOVA analysis for number of BrdU-positive cells in the dentate gyrus

Study	Region	Factor	F-Value	DF	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 x 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 x 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 x Age	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 x Age	5.85	1	18	0.03

GCL = Granular Cell Layer, DF = Degrees of Freedom

Table 2. Phenotype of BrdU-positive cells in the granular cell layer (GCL) and in the hilus of 2, 12, 18 and 24 month-old mice, 4 weeks after the last BrdU-injection.

	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%

BrdU-positive cells were labeled with markers for neurons (NeuN) and astrocytes (S100β). Number of analyzed cells (in parenthesis) and percent of BrdU-colabeled cells are indicated.

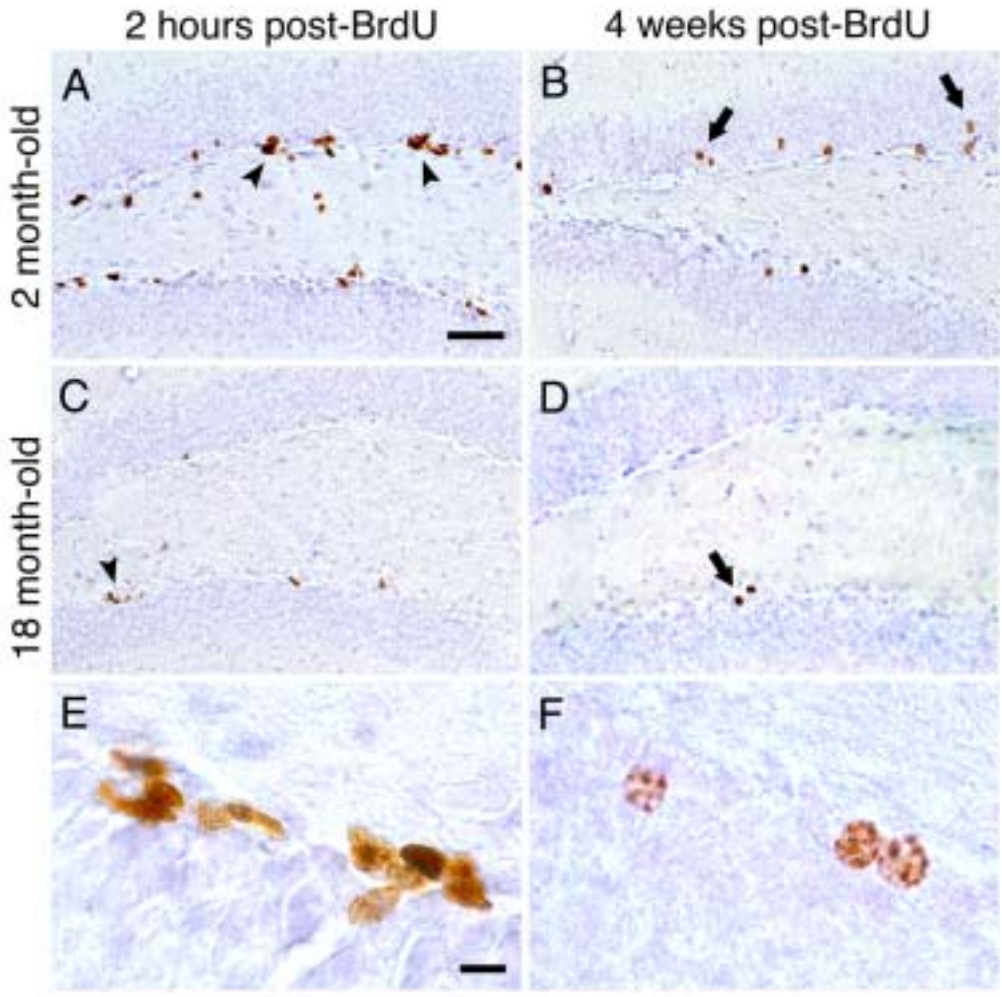
2 hours post-BrdU
4 weeks post-BrdU
2 month-old
18 month-old
A
B
C
D
E
F

Figure 1

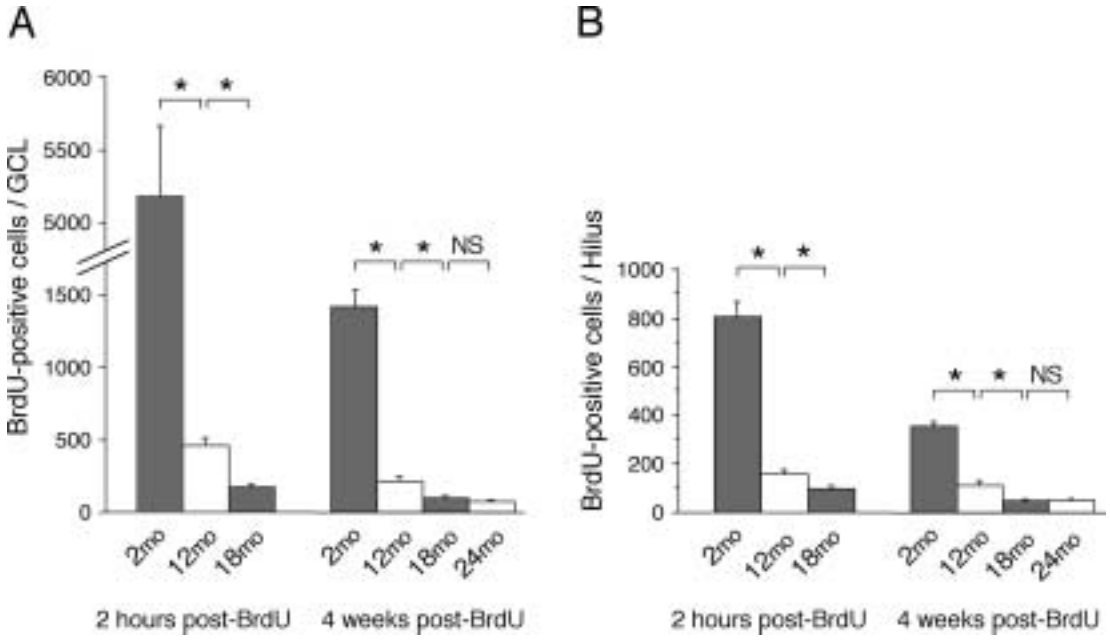
A
B
BrdU-positive cells / GCL
BrdU-positive cells / Hilus
6000
5500
5000
1500
1000
500
0
800
600
400
200
*
NS
2mo
12mo
18mo
24mo
2 hours post-BrdU
4 weeks post-BrdU

Figure 2

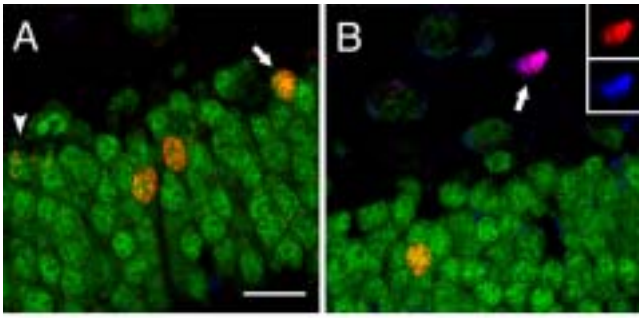

Figure 3

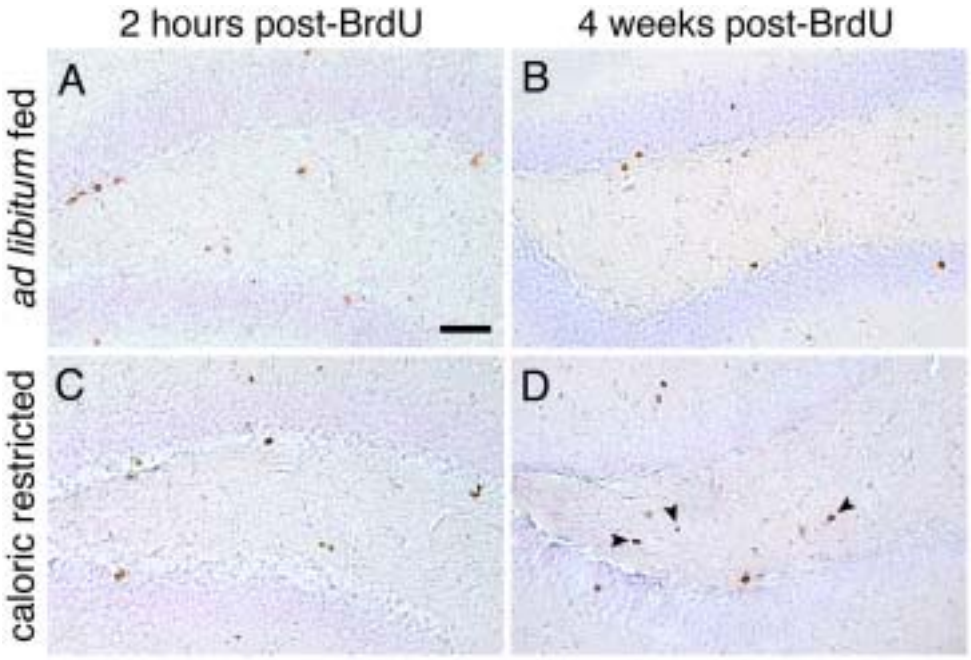
2 hours post-BrdU
4 weeks post-BrdU
A
B
C
D
ad libitum fed
caloric restricted

Figure 4

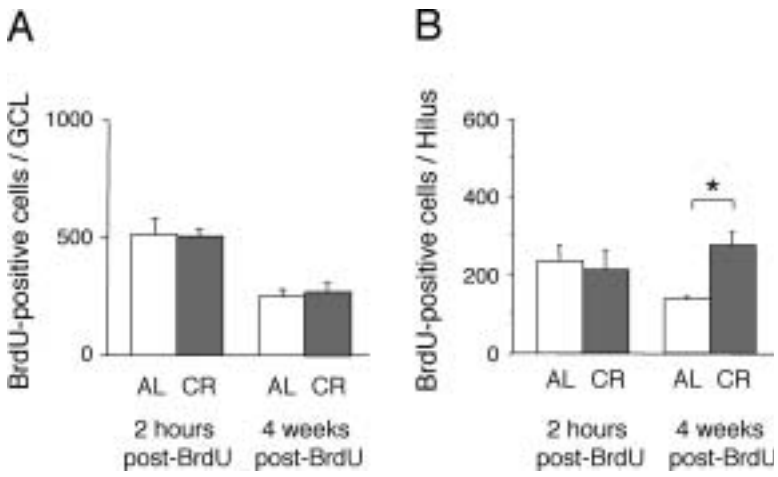
A
BrdU-positive cells / GCL
1000
500
0
AL CR
AL CR
2 hours post-BrdU
4 weeks post-BrdU
B
BrdU-positive cells / Hilus
600
400
200
0
*
AL CR
AL CR
2 hours post-BrdU
4 weeks post-BrdU

Figure 5

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3. AMYLOID-ASSOCIATED NEURON LOSS AND GLIOGENESIS IN THE NEOCORTEX OF AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE

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Summary

APP23 transgenic mice express mutant human amyloid precursor protein and develop amyloid plaques predominantly in neocortex and hippocampus progressively with age, similar to Alzheimer's disease. We have previously reported neuron loss in the hippocampal CA1 region of 14 to 18 month-old APP23 mice. In contrast, no neuron loss was found in neocortex. In the present study we have reinvestigated neocortical neuron numbers in adult and aged APP23 mice. Surprisingly, results revealed that 8 month-old APP23 mice have 13 and 14% more neocortical neurons compared to 8 month-old wildtype and 27 month-old APP23 mice, respectively. In 27 month-old APP23 mice we found an inverse correlation between amyloid load and neuron number. These results suggest that APP23 mice have more neurons until they develop amyloid plaques but then lose neurons in the process of cerebral amyloidogenesis. Supporting this notion, we found more neurons with a necrotic/apoptotic phenotype in the neocortex of 24 month-old APP23 mice compared to age-matched wildtype mice. Stimulated by recent reports that demonstrated neurogenesis after targeted neuron death in the mouse neocortex, we have also examined neurogenesis in APP23 mice. Strikingly, we found a 4-6 fold increase in newly produced cells in 24 month-old APP23 mice compared to both age-matched wildtype mice and young APP23 transgenic mice. However, subsequent cellular phenotyping revealed that none of the newly generated cells in neocortex had a neuronal phenotype. The majority were microglial and to a lesser extent astroglial cells. We conclude that cerebral amyloidosis in APP23 mice causes a modest neuron loss in neocortex and induces marked gliogenesis.

Key words: Alzheimer's disease, amyloid, APP, Aβ, CNS, brain, transgenic mouse, stereology, neurodegeneration, stem cells, neurogenesis, gliogenesis, microglia, aging.

3.1 Introduction

The hallmark lesions of Alzheimer's disease (AD) brain are extracellular deposits of β-amyloid (Aβ) and intracellular neurofibrillary tangles (Probst et al., 1991; Selkoe, 1999). In addition significant neuron and synapse loss in brain regions involved in information processing and memory acquisition have consistently been reported in AD and are thought to be morphological correlates of dementia (West et al., 1994; Gomez-Isla et al., 1996a; Morrison and Hof, 1997).

Considerable effort has been devoted to studying the relationship between amyloid plaques and AD neurodegeneration. In particular, it has remained unclear whether Aβ and/or its deposition in the parenchyma is the cause of the nerve cell loss. While older studies report a poor correlation between dementia and amyloid plaques (Gomez-Isla et al., 1996b; Giannakopoulos et al., 1997), more recent studies have found stronger correlations between either total Aβ load or neuritic Aβ plaques and nerve cell loss and/or dementia (Cummings and Cotman, 1995; Knowles et al., 1998; Naslund et al., 2000).

Several transgenic mouse models of cerebral β-amyloidosis have been generated through expression of mutated amyloid precursor protein (APP) (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Calhoun et al., 1999; Hsia et al., 1999; Lamb et al., 1999; Van Dorpe et al., 2000). These mice develop amyloid plaques and vascular amyloid predominately in neocortex and hippocampus as they age. The amyloid plaques share many features with the amyloid deposits in AD brain. They are surrounded by activated microglia, reactive astrocytes, dystrophic synaptic boutons, and abnormally phosphorylated tau-positive neurites (Masliah et al., 1996; Sturchler-Pierrat et al., 1997; Frautschy et al., 1998; Phinney et al., 1999; Stalder et al., 1999).

To study the impact of cerebral amyloidosis on neurodegeneration, modern stereological techniques have been used to relate neuron number to amyloid burden in these APP transgenic mice (Irizarry et al., 1997a; Irizarry et al., 1997b; Calhoun et al., 1998b; Takeuchi et al., 2000). While in two transgenic mouse lines, Tg2576 and PDAPP mice, no significant nerve cell loss was reported in hippocampus and neocortex (Irizarry et al., 1997a; Irizarry et al., 1997b), we have previously reported that amyloid plaque formation is accompanied by CA1 hippocampal

neuron loss in 14-18 mo old APP23 mice. In contrast, in the same mice we could not detect any global neuron loss in neocortex, despite an amyloid burden comparable to that in the CA1 region (Calhoun et al., 1998b).

The apparent lack of neocortical neuron loss at a given age of APP transgenic mice may, however, be due to compensating mechanisms: In particular, it has been shown that discrete lesions to the neocortex of mice can stimulate neurogenesis (Magavi et al., 2000). It is therefore conceivable that neurogenesis partly compensates for amyloid-associated neuron loss in APP transgenic mice at the lesion sites. Thus, in the present study we have reevaluated neocortical neuron loss and have additionally assessed neurogenesis in the neocortex of adult and aged APP23 mice.

3.2 Materials and Methods

Animals

The generation of APP23 transgenic mice is described elsewhere (Sturchler-Pierrat et al., 1997). Briefly, a murine Thy-1 promoter element was used to drive neuron-specific expression of human APP751 with the Swedish double mutation 670/671KM->NL in B6D2 mice. All mice used were from the F6 to F10 generation of backcrossing to C57BL/6 mice. The wildtype control mice were either littermate mice or nontransgenic age-matched mice from another litter of the same generation of backcrossing.

The following mice were used for neuron counting: Thirteen adult (6 females and 7 males) and 13 aged (7 females and 6 males) APP23 mice. Fifteen adult (7 females and 8 males) and 11 aged (6 females and 5 males) wildtype mice served as controls. All the adult mice were 8 month-old. The aged mice ranged from 26 to 29 months with a mean age of 27 months in both groups. Additional groups of 2 to 3 month-old male APP23 mice (n=6) and male control mice (n=6) were also used for neuron counting. For TUNEL-labeling 4 month-old (n=4) and 24 month-old (n=4) male APP23 mice and equivalent numbers of male age-matched controls were used. For BrdU-labeling 4 month-old (n=4) and 24 month-old (n=8) male APP23 mice and equivalent numbers of male age-matched controls were used.

Histology and immunohistochemistry to assess neuron number and amyloid load

Mice were anaesthetized with 2.5% isoflurane and decapitated. The brains were removed, immersion fixed with 4% paraformaldehyd in PBS for 2 days at 4°C and then embedded in paraffin under standard conditions (Calhoun et al., 1998a). Serial coronal sections were cut at a 25μm microtome setting throughout the entire neocortex. Cresyl violet staining was used for neuron counting. To this end sections were deparaffinized in xylene and rehydrated through a series of graded ethanols. The slides were immersed in warm (60°C) cresyl violet solution (Merck; 0.5g/100 ml in dH2O with 0.3% glacial acetic acid), differentiated in ethanol, cleared in xylene and coverslipped.

To assess amyloid load, sections were deparaffinized in xylene and then placed in 100% ethanol for 10 minutes followed by 30 min in methanol with 0.3% H2O2. Sections were rinsed in PBS and incubated for 1 h in 5% goat serum. Slides were transferred to a humid chamber and sections incubated overnight with a polyclonal antibody to Aβ (NT11) (Sturchler-Pierrat et al., 1997) diluted 1:500 in PBS with 3% goat serum. Sections were then incubated for 60 min with biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:200 in PBS with 3% goat serum, followed by incubation for 90 min in an avidin-biotin-peroxidase complex (Vector Laboratories) diluted 1:200 in PBS. 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. Congo red staining was performed according to standard protocols and was used to assess compact amyloid.

Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL)

Mice were overdosed with pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative overnight and placed in 30% sucrose in PBS for 2 days. Brains were then frozen in 2-methylbutane at -25°C and serially sectioned on a freezing-sliding microtome at 40μm.

The TUNEL assay was performed on free-floating sections using the Apoptag In Situ Cell Death Detection Kit (Intergene, Purchase, NY) according to a recently described procedure (Biebl et al., 2000). In brief, after rinsing sections in TBS for 10 min, an ascending isopropanol series (dH20, 70%, 90% - 2 min each) was followed by incubation in 100% isopropanol for 10 min and a descending isopropanol series (90%, 70%, dH20 - 2 min each). After 3 rinses in TBS, sections were incubated with Equilibration Buffer for at least 5 min at room temperature followed by TdT-reaction solution for 1 hr at 37(C and the Stop Buffer for 10 min at room temperature. To reduce background labeling the TdT-reaction solution was diluted 1:1 with TUNEL Dilution Buffer (Roche Diagnostics, Mannheim, Germany). With intermittent washes in TBS, sections were blocked in 3% donkey serum and 0.1% Triton-X100 in TBS for 30 min. For peroxidase detection, TUNEL-treated sections were incubated with a sheep anti-digoxigenin-FITC antibody (1:1000, Roche Diagnostics, Mannheim, Germany) in TBS overnight at 5(C. TUNEL labeling was visualized by incubation with biotinylated anti-sheep IgG (1:1000, Jackson ImmunoResearch)

followed by the avidin-biotin peroxidase complex (Vector Laboaratories) and diaminobenzidine/ NiCl/H2O2. Sections were counterstained with 1% methylene green and coverslipped.

To study the phenotype of the TUNEL-labeled cells, triple-fluorescence labeling was performed. TUNEL-treated sections were incubated over night at 5(C with combinations of the following primary antibodies: sheep anti-digoxigenin-FITC (see above), mouse monoclonal anti-NeuN (1:1000, Chemicon, Temecula, CA), rabbit polyclonal anti-S100β (1:2000, Swant, Bellinzona, Switzerland), rat anti-CD11b (Mac-1; 1:1000, Serotec, Oxford, UK). After intermittent washes in TBS and brief fixation in 4% paraformaldehyde (15 min), primary antibodies were detected using combinations of the following secondary antibodies: FITC-labeled donkey anti-sheep IgG (to enhance the sheep anti-digoxygenin-FITC signal), RhodamineX-labeled donkey anti mouse IgG, RhodamineX-labeled donkey anti rat-IgG, CY5-labeled donkey anti-rabbit IgG (all at 1:500 for 2hrs; Jackson ImmunoResearch, West Grove, PA). 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 with bleach protection (SlowFade; Molecular Probes, Eugene, OR) or Vectashield (Vector Laboratories). Sections were analyzed with a Confocal Laser Scanning Microscope LSM 510, inverted Axiovert 100 M (Zeiss).

BrdU labeling and cellular phenotyping

For labeling of newly generated cells mice were given daily injections of bromodeoxyuridine (BrdU; 50μg/g body weight i.p., Sigma) for 5 consecutive days. One day or four weeks after the last BrdU application mice were overdosed with pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative overnight and placed in 30% sucrose in PBS for 2 days. Brains were then frozen in 2-methylbutane at -25°C and serially sectioned on a freezing-sliding microtome at 40μm.

Sections were pretreated in 50% formamide in 2x SSC for 2 hrs at 65°C, followed by 10 min in 2x SSC, 30 min in 2N HCl at 37°C and 10 min in 0.1M borate buffer. Sections were then incubated in 0.08% H2O2, 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. The chromogen was Vector SG (Vector Laboratories).

To study the cellular phenotype of BrdU-labeled cells, double and triple immunofluorescence stainings were performed with a combination of antibodies to NeuN, S100β, and CD11b (see above). The secondary antibodies were Alexa488 goat anti-mouse IgG, Alexa 568 goat anti-rat IgG and Alexa633 goat anti-rabbit IgG (1:400, Molecular Probes). CD11b/BrdU double staining was performed sequentially, i.e. sections were first reacted for CD11b, followed by BrdU-pretreatment and detection. In case of high autofluorescence background, sections were treated with Sudan Black B (see above). Sections were analyzed with a Confocal Laser Scanning Microscope (see above).

Stereological analysis

Neocortical neuron number was estimated on paraffin embedded cresyl violet stained-sections and amyloid load was estimated on Aβ-immunostained paraffin sections as previously reported (Calhoun et al., 1998b). Briefly, for both neuron number and plaque load quantification, a systematic random series of every 20th section throughout the entire neocortex was selected, yielding 10-15 sections per animal. Quantification of neuron number was done by first estimating the volume of the neocortex by the Cavalieri point-counting method (grid point area=500μm2; mean number of grid points 97±2.4). Numerical density of neurons was then estimated by counting the number of topmost neuronal nucleoli (using a 100x, 1.3NA objective; on-screen magnification: 2759x) within three-dimensional optical disectors that were systematic-randomly spaced throughout the neocortex (area=752μm2, height=14μm, guard height=4μm; mean number of disectors sampled=81±1.8; mean number of neurons counted per disector = 2.2±0.05). Only cells with typical neuronal morphology, including a clear nucleolus were counted. Sampling was optimized to produce a coefficient of error under the observed biological variability. The product of volume times numerical density was calculated to estimate total neuron number. Anatomical regions were defined according to the Franklin & Paxinos mouse brain atlas (Franklin and Paxinos, 1997), and reliable anatomical boundaries were established at all levels (neocortex borders: olfactory bulb/tubercle, corpus callosum/ external capsule, pia mater, endopiriform nuclei, claustrum, amygdala and subiculum). Postprocessing section thickness was measured at

each disector location using a focus drive with ±0.1μm accuracy (Applied Scientific Instrumentation, Eugene, OR). The mean section thickness was 26.1±0.6. Results reflect numbers for the right hemisphere only. Plaque load was estimated by calculating the area fraction occupied by amyloid in two-dimensional disectors on a single focal plane (20x objective, 0.45NA) (Calhoun et al., 1998b). The percentage of diffuse amyloid was calculated on sections double stained for Aβ and Congo red and examined under cross-polarized light. Diffuse amyloid was defined as Aβ-positive and Congo red-negative. The number of TUNEL-positive cells was counted in 7-8 sections throughout the neocortex. Using a 40x, 0.75NA objective, TUNEL-positive cells/section were counted. Since sections were not systematically sampled, no estimation of total cortical TUNEL-positive- cells per hemisphere was performed. The number of BrdU-labeled cells was determined on systematic random series of every 12th section throughout the entire neocortex (12-16 fixed-frozen sections per animal). All BrdU- labeled neocortical cells were counted using a 40x, 0.75NA objective. Total number was calculated by multiplying the number of counted cells times the section interval 12. The results reflect numbers for the right hemisphere only. In contrast to neuron counting, no guard height was included. For reasons of counting efficiency we have not excluded this potential source of error, because qualitative analysis indicated a very robust difference in number of BrdU-labeled cells among the groups.

All stereological analysis was performed with the aid of StereologerTM software and a motorized x-y-z stage coupled to a video-microscopy system (Systems Planning and Analysis, Inc., Alexandria, VA). All brains were processed and analyzed in batches of four (aged transgenic, young-adult wildtype, aged wildtype, young-adult transgenic) to minimize methodological errors. Results were analyzed using ANOVA with the help of StatView 5.0.1 Indicated is the mean and the standard error of the mean. The level of significance was set at≤0.05.

3.3 Results

Amyloid load and neural cytoarchitecture in APP23 mice

Eight month-old APP23 mice exhibited only few amyloid plaques in the neocortex (Fig. 1A). Typically, they first appeared in the frontal cortex, were very small, and of compact and congophilic nature. Diffuse amyloid was not observed in 8 month-old mice. Quantitative analysis revealed that the volume fraction occupied by amyloid ranged from 0.1 to 0.4%. In contrast, an amyloid load of 15.9 to 28.0% (mean 24.1 ± 0.9%) was found in 27 month-old APP23 (Fig. 1B). In these aged mice, diffuse amyloid represented 30-40% of the total amyloid load and showed a considerable region-specific variability.

Cresyl violet staining revealed that amyloid deposits severely disrupt neuronal cytoarchitecture in the neocortex (Fig. 2). In 27 month-old APP23 mice cortical layers were often barely detectable and layer V pyramidal cells appeared in islets in between amyloid plaques with dendrites and axons leaving the cells in various directions. At higher magnification, neurons appeared displaced by the growing amyloid deposits, giving the impression of a higher density of neurons between amyloid plaques. However, at the intimate amyloid plaque periphery, neurons were largely missing. In contrast, a layer of glial cell nuclei appeared clustered around the amyloid (Fig. 2D). We have previously shown that most of these glia cells are microglia with their processes forming an intimate relationship with the amyloid fibrils (Stalder et al., 1999; Stalder et al., 2001)

Estimation of total neocortical neuron number in 8 and 27 month-old APP23 mice

Using stereological methods we have quantified neurons in adult and aged, 8 and 27 months, APP23 mice and in corresponding controls. ANOVA for total number of neurons per hemisphere revealed significant effects for the three main factors age ($F(1,44)=7.53$; $p<0.01$), genotype ($F(1,44)=6.48$; $p<0.05$), and sex ($F(1,44)=13.52$; $p<0.001$). The observation that in all groups males had on average 10% more neurons than did females was confirmed by a lack of further interactions with the factor sex. In contrast, a significant interaction was found between age and genotype ($F(1,44)=5.28$, $p<0.05$). Subsequent post-hoc analysis (Fisher's test) revealed that the 8 month-old APP23 mice had modest but significant 13% to 15% increases in neocortical neuron number compared to 8 month-old wildtype mice, to 27 month-old wildtype mice but also

to 27 month-old APP23 mice (all p's<0.01) (Fig. 3A). No age-related loss of neocortical neuron number was found in the control mice.

This surprising observation indicates that APP23 mice have more neurons than do wildtype mice before they develop significant cerebral amyloidosis but then lose neurons with further aging to reach the levels of wildtype mice at 27 months of age. To sustain this interpretation and because 27 month-old APP23 mice show great variability in amyloid load (see above), we wondered whether amyloid load predicts neuron number in the 27 month-old APP23 mice. Indeed, linear regression analysis (Fig. 3B) revealed a significant inverse relation between neocortical neuron number and amyloid load in the 27 month-old APP23 mice ($R^2(11)=0.55$, $p<0.01$).

To determine whether the increase in neuron number in 8 month-old APP23 mice occurs during or after brain development we have assessed neocortical neuron number in an additional group of young 2-3 month-old male APP23 mice and littermate control mice. Interestingly, we found also in these young APP23 mice 10% more neocortical neurons compared to non-transgenic littermates (6.31E6±0.16E6 vs. 5.79E6±0.17E6; $t(10)=2.22$, $p=0.05$). The absolute neuron numbers in these young groups is higher compared to the adult and aged mice because only male mice were used.

Amyloid-associated cell death

Analysis of cresyl violet stained sections of 24 month-old APP23 mice occasionally revealed neurons with a pyknotic appearance and an irregular membrane structure. Such cells were in most cases in the vicinity of amyloid plaques (Fig. 4A). To identify nuclear profiles with DNA fragmentation, one of the hallmarks of apoptosis, we then used the TUNEL-labeling technique. Results revealed a nearly fourfold increase in TUNEL-positive cells/section in 24 month-old APP23 mouse neocortex compared to age-matched wildtype mice (7.2±0.65 vs. 1.9±0.27; $p<0.001$). The majority of these TUNEL-positive cells was clearly localized to amyloid plaques (Fig. 4B). We have also used immunohistochemistry using CM-1 antibody (provided by A. Srinivasan) to activated caspase-3, which is thought to be an important step in apoptotic cell death (Nicholson et al., 1995). Again we found more CM-1 positive cells in aged APP23 mice compared to wildtype mice (results not shown).

To establish the nature of these apoptotic cells, double labeling for TUNEL and NeuN, S100β, and CD11b was used (Fig. 4C-E and Table 1). However, most of the TUNEL-positive cells (77%) did not colocalize with any of these markers. Six percent were NeuN-positive and could be identified as neurons. Unexpectedly, 17% of the TUNEL-positive cells expressed CD11b and were identified as microglia. None of the TUNEL-positive cells expressed the astroglial marker S100β. The observation that the vast majority of the TUNEL-positive cells could not be identified with cell-type specific markers, may be because these cells are in late stages of apoptosis (Biebl et al., 2000).

Increase in BrdU-positive cells in aged APP23 mice

Previous work has shown that apoptotic cell death can induce neurogenesis in the mouse neocortex (Magavi et al., 2000). For this reason we have studied the generation of new cells in the neocortex of 4 and 24 month-old APP23 mice and age-matched wildtype control mice.

In a first experiment mice were sacrificed 4 weeks after the last BrdU injection to allow newly produced cells to differentiate into their final cell type (Gage, 2000). Results revealed a striking increase in BrdU-positive cells in aged APP23 mice compared to aged wildtype mice with most of the labeled cells around the amyloid deposits (Fig. 5A,B). Quantitative analyses (Fig. 6) confirmed that 24 month-old APP23 mice have a significant 4-6 fold increase in BrdU-labeled cells (ANOVA age x genotype: $F(1,12)= 46.5$; $p<0.001$) in comparison to 24 month-old wild-type mice and to 4 month-old APP23 ($p's<0.001$).

To determine whether the newly generated cells in aged APP23 mice are in fact neurons, BrdU-labeling was combined with the neuronal marker NeuN (Fig. 5C; Table 1). However, although 240 BrdU-positive cells (60/mouse) have been studied for colocalization with NeuN, we could not detect any neocortical colabeled cell. Subsequent colabeling with S100β and CD11b revealed 58% of the labeled cells as microglia and 16% as astrocytes, while the remaining cells could not be allocated to one of these cell types (Fig. 5D,E; Table 1).

We have also studied an additional group of 24 month-old APP23 mice and age-matched wild-type control mice that were sacrificed one day after the last BrdU injection. A significant nearly 4-fold increase in BrdU-positive cells in APP23 neocortex was detected, when compared to

control mice (5.21E4±1.0E4 vs. 1.47E4±2.3E3; t-test: p=0.01). This increase is very similar to that observed in 24 month-old APP23 mice sacrificed 4 weeks after the last BrdU-injection. These results favor the hypothesis that glial proliferation is upregulated in APP23 mice. However, an effect on cell survival should not be ruled out since BrdU was injected over 5 days and considerable death of newly generated cells may have occurred within this period.

3.4 Discussion

In the present study we have reinvestigated neocortical neuron loss in APP transgenic mice by comparing two large homogeneous groups of adult and aged APP23 mice of both genders. For the adult group we chose 8 month-old mice, an age at which cerebral amyloidosis starts to develop. It has previously been shown that aging renders the brain vulnerable to A(toxicity (Geula et al., 1998) and thus we used aged APP23 mice that were 26-29 months-old, which is beyond the mean life span of C57BL/6 mice (Jucker and Ingram, 1997).

The present estimate of total number of neurons in the mouse neocortex was about 5.5E6. This result is consistent with our previous estimate (Calhoun et al., 1998b). Furthermore, the present findings show that males have a robust 10% increase in neuron number compared to females. This observation is interesting in view of a 16% gender difference that has been reported in humans (Pakkenberg and Gundersen, 1997). In wildtype murine neocortex we did not find an age-related neuron loss while in humans, albeit with a larger cohort, a 10% neuron loss with aging was reported (Pakkenberg and Gundersen, 1997).

Most significant for the present investigation we have found that young and adult APP23 mice have 10-15% more neurons compared to wildtype control mice. This observation was surprising, but in light of previous results not entirely unexpected. APP and/or its secreted form sAPP have been implicated in cell growth and cell survival, and in the protection of neurons against excitotoxicity (Saitoh et al., 1989; Milward et al., 1992; Mattson et al., 1993; Roch et al., 1994; Smith-Swintosky et al., 1994; Perez et al., 1997). An increased number of synapses and augmented neuroprotection to excitotoxic injuries has also been reported in APP transgenic mice (Mucke et al., 1994; Masliah et al., 1997). Similarly, we have recently found an increase in the number of synapses in adult APP23 transgenic mice compared to control mice (unpublished observations). The present observation of an increased neocortical neuron number in adult APP23 mice adds to the evidence that APP overexpression in transgenic mice has growth-promoting and neuroprotective features. If this increase in neuron number is a more general phenomenon of APP overexpression it may also occur in hippocampus of APP23 mice. This, in turn, raises the possibility that the neuron loss in CA1 of APP23 mice is in fact greater than previously reported (Calhoun et al., 1998a). Although APP overexpression is a likely cause, it cannot be ruled out

that the increase in neuron number in APP23 mice is an effect of the transgene insertion site. Thus, it will be important to replicate our observation in other APP transgenic mice with similar genetic backgrounds and APP expression levels. In addition, it may be interesting to examine whether the overexpression of APP in Down's Syndrome also leads to an increased neuron number.

Given that APP23 mice have 10-15% more neocortical neurons when they start to develop cerebral amyloidosis, our results suggest that they lose about this percentage of neurons in the course of cerebral amyloidogenesis. The neuron loss in aged APP23 mice is in line with the increased appearance of neurons with a necrotic and/or apoptotic phenotype and with the inverse correlation observed between neuron number and amyloid load.

In two other transgenic mouse lines, Tg2576 mice and PDAPP mice, no significant nerve cell loss was reported in hippocampus and neocortex (Irizarry et al., 1997a; Irizarry et al., 1997b). This difference compared to the APP23 mouse line may be due to the lower amyloid burden reported in Tg2576 mice (Irizarry et al., 1997b) and the more diffuse nature of amyloid deposits in PDAPP mice (Masliah et al., 1996; Irizarry et al., 1997a). Neuron number in the frontal cortex has recently also been assessed in Tg2576 mice that were crossed with mutant PS1-overexpressing mice. Such mice also reveal a high (compact) plaque load, similar to that in APP23 mice. However only 1-4 mice were analyzed per group, and thus a 15% loss may not have been detectable (Takeuchi et al., 2000). Furthermore, we have reported age-dependent hemorrhagic stroke in APP23 mice, which may contribute to neurodegeneration (Winkler et al., 2001).

The neuron loss in neocortex of APP23 mice appears modest, but in fact exceeds the 2-6% global neuron loss reported in AD neocortex using similar stereological methodology (Regeur et al., 1994; Bundgaard et al., 2001). In contrast, 32% neuron loss has been reported in AD if the entorhinal cortex is analyzed separately, and up to 90% neuron loss was observed when individual laminae of the entorhinal cortex were analyzed, emphasizing that the neuropathic manifestations of AD are region-specific (Gomez-Isla et al., 1996a). Unfortunately, entorhinal cortex neuron number could not be assessed in APP23 mice because the massive amyloid deposition in aged APP23 prevented the reliable identification of the anatomical borders of the entorhinal cortex, an essential prerequisite for unbiased quantification. Nevertheless, qualitative

neuron loss is also clearly observed in the entorhinal cortex of aged APP23 mice, although it probably does not reach the extent reported in humans (Calhoun et al., 1998b). Consistently, it has been reported that Aβ neurotoxicity in vivo is species-dependent with a much higher toxicity in primates compared to rodents (Geula et al., 1998). Moreover, APP overexpression per se is neuroprotective against amyloid-induced neurotoxicity (as discussed above). Thus, it will be important to assess neurodegeneration in mouse models of cerebral amyloidosis that do not overexpress APP (Iwata et al., 2000; Popp et al., 2000).

The view that estimation of total neuron numbers is suited to determine the extent of neuronal degeneration has recently been challenged by the observation of neurogenesis in mouse and rat neocortex after targeted apoptotic lesions and focal cerebral ischemia (Gu et al., 2000; Magavi et al., 2000). In one of these studies, it was demonstrated that some of the newly produced neurons in the vicinity of the lesion site extend axons into the denervated region and thus, appear to replenish damaged neuronal circuits (Magavi et al., 2000). These observations, together with recent findings that overexpression of APP may enhance proliferation of neural stem cells (Ohsawa et al., 1999) and that neocortical stem cells have the potential to differentiate into neurons in vitro (Palmer et al., 1999), suggest that neocortical neuron number in APP23 transgenic mice might be viewed as the result of the dynamic equilibrium achieved between the continuous loss and birth of neurons.

However, in the present study we found no evidence for neurogenesis either in wildtype mice (confirming previous studies, e.g. Kuhn et al., 1997) or in APP23 mice with a massive amyloid load. Because we have counted a total of 240 cells in four mice we cannot exclude that neurogenesis occurs with a prevalence lower than 0.5%. In contrast, 1-2% and 3-6% of BrdU-positive cells colocalized with NeuN after targeted apoptosis and ischemic stroke, respectively (Gu et al., 2000; Magavi et al., 2000). Thus, we conclude that neurogenesis does not occur, or is an extremely rare event, in response to cerebral amyloidosis in APP23 mice. It will be important to determine whether the same is true in AD.

The finding that 58% of the BrdU-labeled cells were in fact microglia substantiates and extends our previous preliminary observation (Bornemann et al., 2001) and indicates a significant number of newly produced microglial cells predominantly around amyloid plaques. Because BrdU has a half life of about 2 hours (Phuphanich and Levin, 1985) and was injected once daily for 5 days, we estimate that about 2 million new microglia are produced per month in the neocortex of aged APP23 mice. At present it is not clear whether these newly produced microglia are the product of mitotic microglia or recruited macrophage. It could even be considered that they originate from neural stem cells. Furthermore, the fate of amyloid-associated microglia is not well understood. Using DNA fragmentation labeling in situ, degenerating microglial cells have been previously described around amyloid in the neocortex of AD patients (Lassmann et al., 1995). Similarly, results of the present study suggest apoptotic microglial cell death around amyloid plaques in APP23 mice. It is tempting to speculate that the continuous production and death of microglia play an important role in cerebral amyloidosis and in AD.

In conclusion, the present results demonstrate that cerebral amyloidosis in the neocortex of aged APP23 trangenic mice causes a modest but significant neuron loss and marked gliogenesis. The contribution of these changes to the reported cognitive impairment of APP23 mice (Kelly et al., 1999) and for AD pathogenesis and therapy remains to be evaluated.

3.5 Figure Captions

Figure 1:Cerebral amyloidosis in the neocortex of APP23 transgenic mice. (A) In 8 month-old mice only few Aβ-immunostained amyloid deposits were found (arrows). (B) In contrast, 27 month-old APP23 mice exhibited severe cerebral amyloidosis throughout the neocortex. The total amyloid load in the neocortex of the mouse shown was estimated to be 25.7%. Scale bar is 150μm. A and B have the same magnification.

Figure 2: Amyloid plaques disrupt neocortical cytoarchitecture in aged APP23 mice. (A) Cresyl violet staining in the neocortex of a 27 month-old wildtype mouse reveals the typical cortical cell layers. (B) In contrast, in 27 month-old APP23 mice amyloid deposits disrupt the neurocytoarchitecture, and some of the layers are barely detectable. (C,D) Higher magnifications of layer V neurons in a 27 month-old wildtype (C) and transgenic (D) mouse. Note the numerous glial cell nuclei (arrows in D) and the absence of neurons in the immediate vicinity of the amyloid plaques. Bars are 150μm (A,B) and 40μm (C,D).

Figure 3: Stereological estimation of total neocortical neuron number. (A) Total neuron number per neocortex per hemisphere in 8 and 27 month-old APP23 and wildtype control mice. For this graph males and females were combined (n=11-15 per group). Results revealed that 8 month-old APP23 mice have more neurons compared to the three other groups ($p<0.01$). (B) For the 27 month-old APP23 mice, linear regression analysis revealed an inverse relation between neuron number and amyloid load.

Figure 4: Cell death with necrotic and apoptotic appearance in the vicinity of amyloid plaques. (A) Neurons with a pyknotic appearance and an irregular membrane structure (arrows) were occasionally detected in 24 month-old APP23 mice. Such neurons were almost exclusively associated with amyloid deposits. (B) TUNEL-positive cells (arrowheads) in the vicinity of an amyloid plaque. Using confocal microscopy TUNEL-positive cells (red) were labeled with markers for either neurons (C), microglia (D) or astrocytes (E) (all in green). Most of the cells could not be phenotyped. Only occasionally were TUNEL-positive cells labeled for NeuN or CD11b. No double labeling was observed with S100β (see Table 1). The insert in C represents single confocal sections of both markers. Bars are 40μm (A,B) and 10μm (C-E).

Figure 5 : BrdU-positive cells in the neocortex of aged APP23 mice. (A) Only few labeled cells were observed in the neocortex of 24 month-old wildtype mice four weeks after BrdU injections. (B) In contrast, there was a nearly fourfold increase in labeled cells in age-matched APP23 mice (for quantitative results see Figure 6). Most of these cells appeared to be associated with amyloid plaques (arrowheads). To study the cellular phenotype of these cells BrdU immunofluorescence (red) was combined with NeuN (C) CD11b (D) and S100β (E) (all in green) and colocalization was assessed with confocal microscopy. None of the BrdU-labeled cells were positive for NeuN (C). In contrast, the majority of the BrdU-labeled cells revealed colocalization with the microglia marker CD11b (D). Colocalization was also found with the astrocytic marker S100β (E). The insert in (E) represents single confocal sections of both markers. Quantitative phenotyping is summarized in Table 1. Bars are 75μm (A,B) and 10μm (C-E).

Figure 6: Increase in BrdU-positive cells in the neocortex of aged APP23 mice. Total BrdU-positive cells in the neocortex of young (4 month-old) and aged (24 month-old) wildtype and APP23 mice. Numbers are for one hemisphere only. Note the nearly fourfold increase in BrdU-labeled cells in the aged APP23 mice compared to all three other groups (p's<0.01).

Table 1
Phenotype of TUNEL- and BrdU-positive cells in neocortex of 24 month-old APP23 mice

	TUNEL		BrdU	
	# Cells analyzed	% Cells colabeled	# Cells analyzed	% Cells colabeled
NeuN	87	6	240	0
S100ß	102	0	240	16
CD11b	102	17	120	58

TUNEL- and BrdU-positive cells were labeled with markers for either neurons (NeuN), astrocytes (S100ß) or microglia (CD11b). Number of analyzed cells and percent of TUNEL- or BrdU-colabeled cells are indicated.

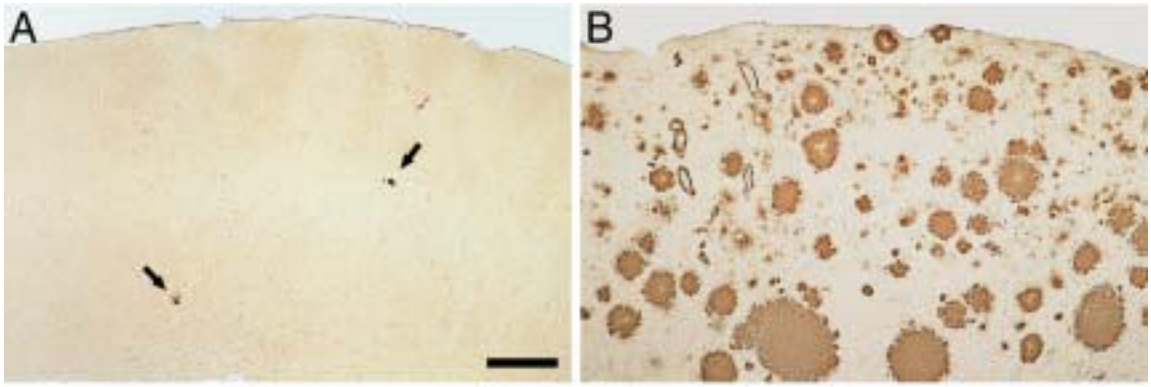

Figure 1

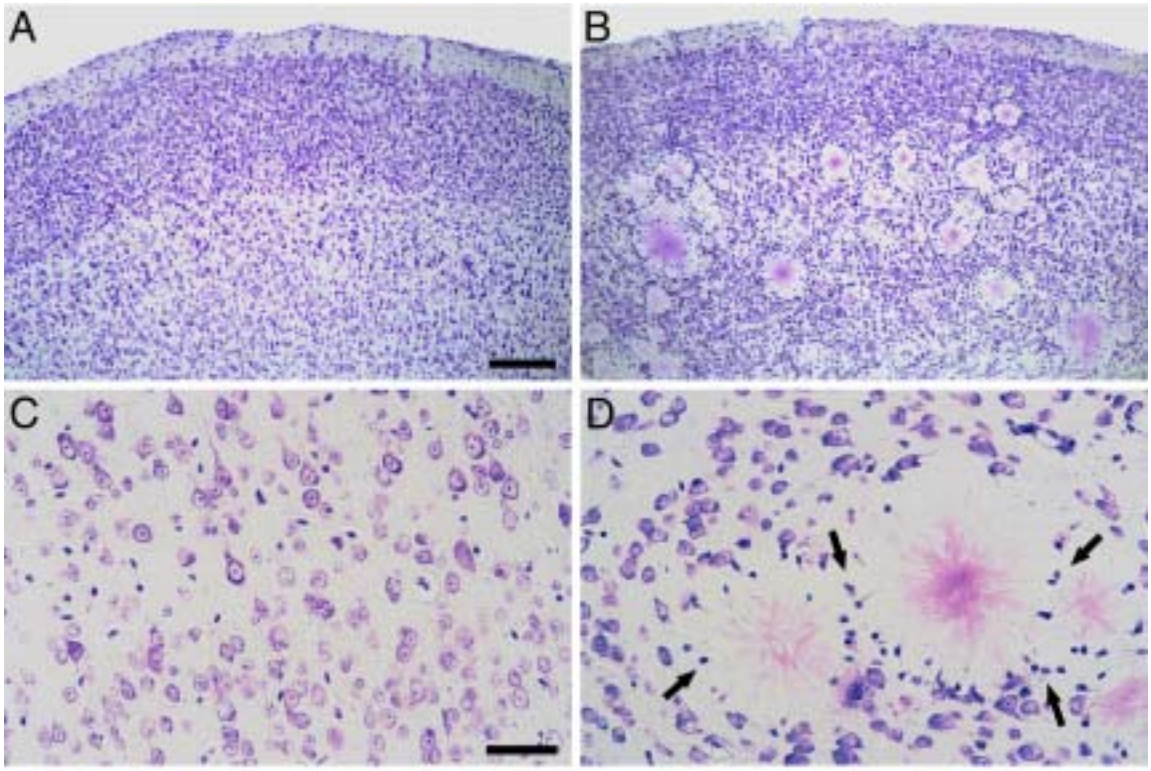

Figure 2

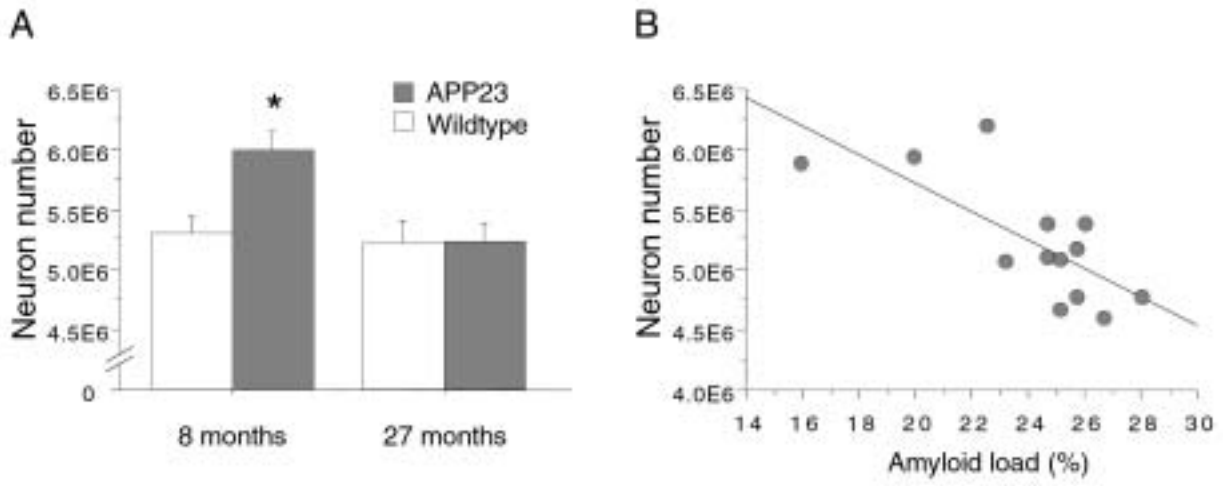
A
B
Neuron number
6.5E6
6.0E6
5.5E6
5.0E6
4.5E6
0
APP23
Wildtype
8 months
27 months
4.0E6
14
16
18
20
22
24
26
28
30
Amyloid load (%)

Figure 3

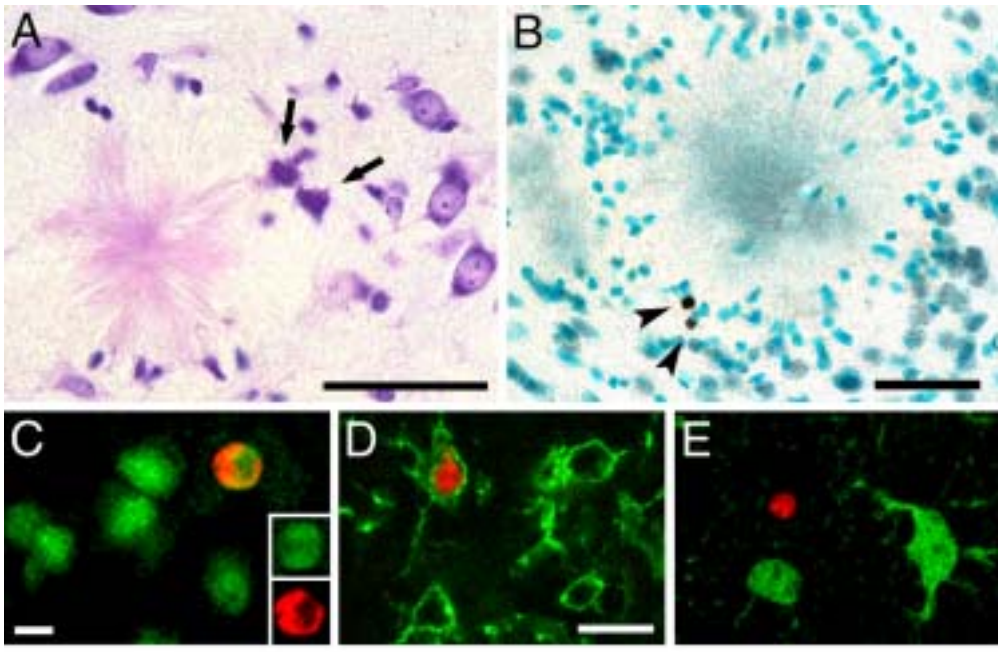

Figure 4

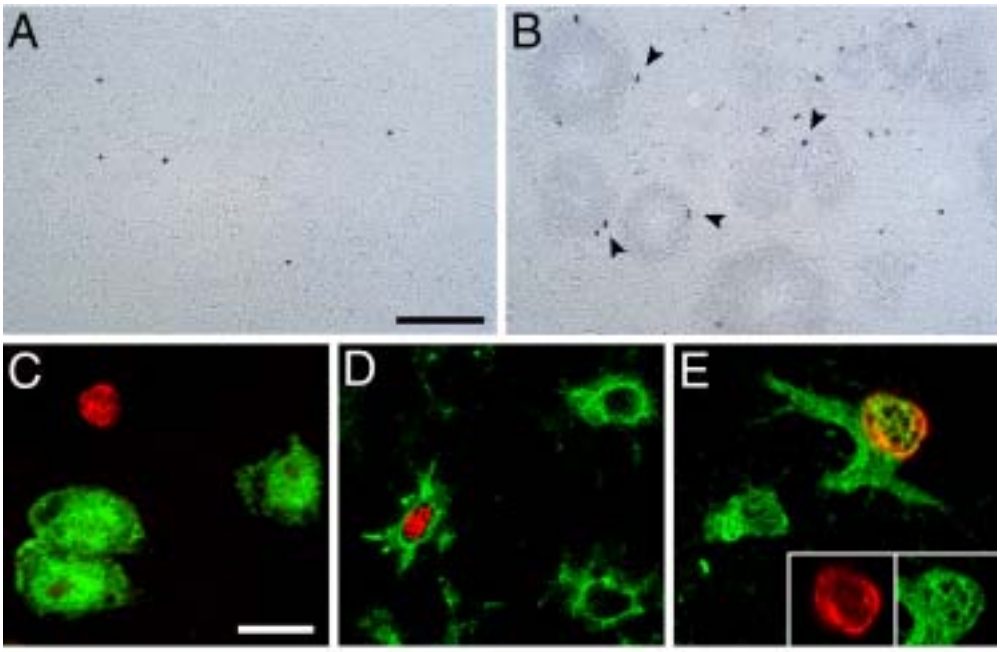

Figure 5

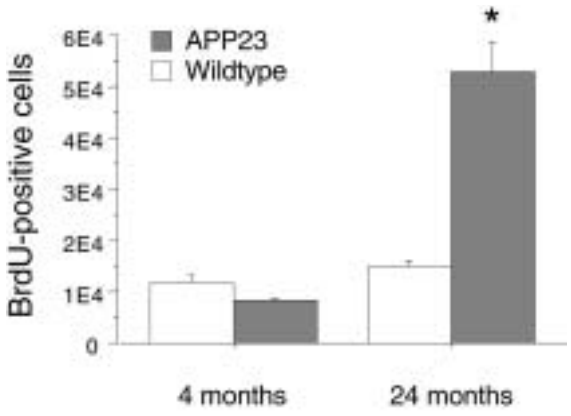
BrdU-positive cells
6E4
5E4
4E4
3E4
2E4
1E4
0
APP23
Wildtype
*
4 months
24 months

Figure 6

3.6 References

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4. CEREBRAL HEMORRHAGE AFTER PASSIVE ANTI-ABETA IMMUNOTHERAPY

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Immunotherapy for Alzheimer's disease (AD) has been the subject of intense investigation. Both active and passive immunization against β-amyloid peptide (Aβ) in mouse models reduce levels of Aβ, prevent and clear amyloid plaques, and improve cognitive behavior[1]. We studied passive immunization of APP23 transgenic mice, a model that exhibits the age-related development of amyloid plaques and neurodegeneration as well as cerebral amyloid angiopathy (CAA) similar to that observed in human AD brain[2,3]. Consistent with earlier reports, we found that passive Aβ-immunization results in a significant reduction of mainly diffuse amyloid. However, it also induces an increase in cerebral microhemorrhages associated with amyloid-laden vessels, suggesting a possible link to the neuroinflammatory complications of Aβ-immunization recently seen in human trial[1].

Male 21 month-old APP23 mice (n=10) were passively immunized weekly by intraperitoneal injections of 0.5 mg β1 mouse monoclonal IgG1 antibody that recognizes amino acids 3-6 of human Aβ[4]. Age-matched APP23 control mice were injected with PBS (n=11). After 5 months of treatment, stereological analysis of amyloid load[4] revealed a significant amyloid reduction (23%; p=0.0008) in neocortex of the immunized mice compared with controls (Fig. 1A,B). This reduction was largely accounted for by a reduction (33%; p=0.001) in diffuse amyloid. ELISA measurements of formic acid extracted brain samples[4] showed a significant reduction in $A\beta_{42}$ (44.8±2.7 and 34.7±3.1 ug/g wet weight for control and immunized mice, respectively; p=0.03) but no significant reduction in $A\beta_{40}$ (166.2±11.3 vs. 152.7±12.2 ug/g).

CAA frequency and severity[4] were not affected by immunization. However, immunized mice exhibited a more than twofold increase in the frequency of CAA-associated cerebral hemorrhage as well as a significant increase in hemorrhage severity over controls (Fig. 1C-J). Most hemorrhages could clearly be attributed to amyloid-laden vessels, and bleedings only occurred in brain areas affected with CAA. Moreover, we found six acute hematomas in immunized compared with only one hematoma in control mice. Similar immunization of 6 month-old APP23 mice (n=12), which exhibited modest parenchymal amyloid but lacked significant CAA, revealed no hemorrhages.

Deposition of amyloid in cerebral blood vessels leads to a loss of smooth muscle cells and a weakening of the vessel wall in mice and humans[5,6]. Our findings suggest that passive Aβ-immunization increases the risk of cerebral hemorrhage by further weakening of the amyloidotic

vessel wall. A potential mechanism is that antibody binding to vascular amyloid triggers a local inflammatory reaction, which might be sufficient to destabilize the already weakened vessel wall[6]. A link between AD-type vascular pathology and inflammation has been suggested[7]. Alternatively, antibody binding to soluble Aβ in blood may lead to increased vascular permeability with a concomitant invasion of plasma proteins and diapedesis that in turn may increase the risk of hemorrhage[6,8]. We found no evidence for involvement of the extrinsic coagulation cascade , although we have previously reported that thrombolytic treatment enhances hemorrhagic diathesis in APP23 mice[4].

No adverse side effects have been reported in other mouse Aβ-immunization paradigms; however, the mouse models used in those studies do not develop significant CAA[1]. As over 10% of people beyond 65 years of age and 80% of AD cases exhibit CAA[9,10], anti-Aβ immunotherapy protocols may be best developed in mouse models that show CAA in addition to brain parenchymal amyloid deposits. Although the anti-NH_2-terminal Aβ antibody used in this study resulted in an increase in CAA-associated microhemorrhages, further screening of antibodies that recognize other Aβ epitopes or conformations may identify antibodies that do not have this effect. Although difficult to diagnose pre-mortem[10], our results also suggest that the success of Aβ-immunotherapy may be improved by screening AD patients for the presence and severity of CAA before such therapies are undertaken.

Figure Caption

Figure 1: Amyloid pathology in the neocortex of a control (A) and an age-matched immunized APP23 mouse (B). Hemosiderin staining reveals an increased number of microhemorrhages (arrowheads) in the immunized (D) compared with control mice (C). Hemorrhages in the immunized mice often reached considerable sizes (E). Double staining for hemosiderin (blue) and Aβ (brown) demonstrates that most bleedings were associated with amyloid-laden vessels (F). H&E staining reveals a fresh bleed in an immunized mouse (G). Quantification[4] of microhemorrhage frequency per unilateral neocortex (H) revealed a more than twofold increase in immunized (imm) compared with control (ctr) mice (*p=0.02). Hemorrhage severity (I) and hemorrhage score (J) also showed significant increases (**p=0.003 and *p=0.01). Scale bars are 100μm (A-D), 100μm (E), 20μm (F), and 200μm (G).

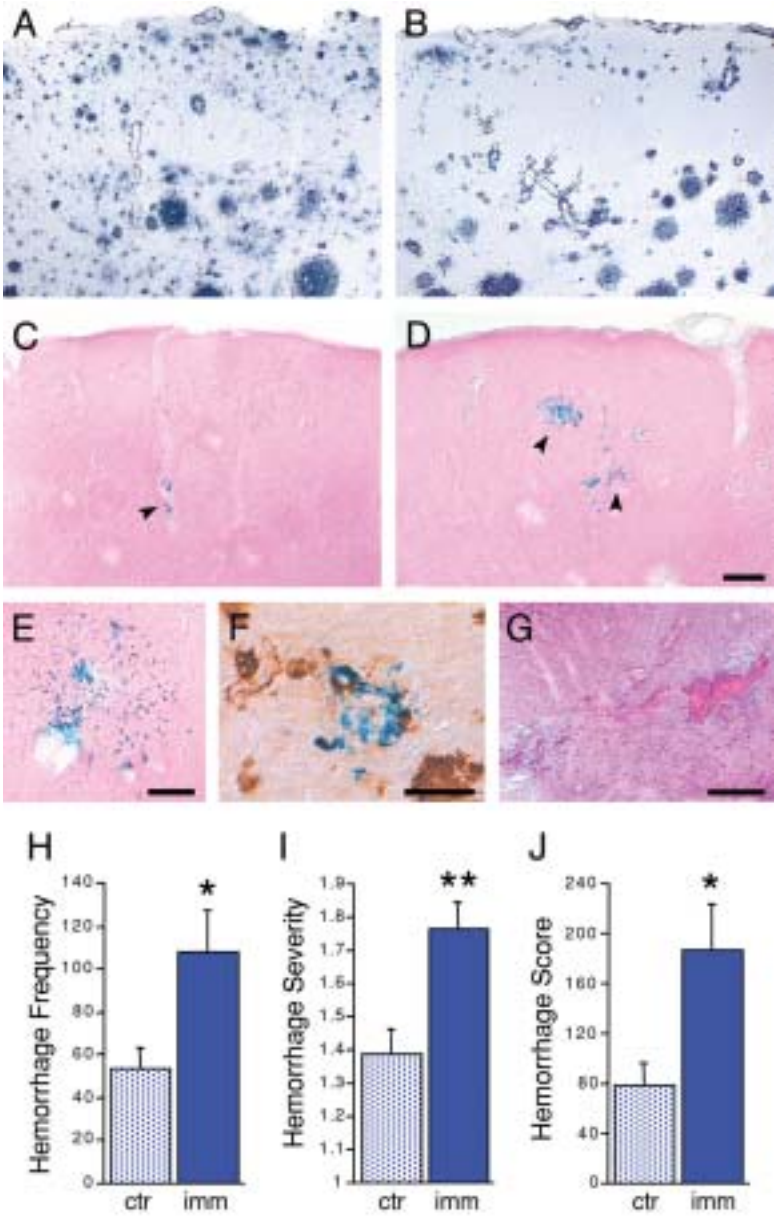
A
B
C
D
E
F
G
H
Hemorrhage Frequency
140
120
100
80
60
40
20
0
*
ctr
imm
I
Hemorrhage Severity
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
**
ctr
imm
J
Hemorrhage Score
240
200
160
120
80
40
0
*
ctr
imm

Figure 1

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Supplementary Material

Animals and Immunization

APP23 mice[1] were passively immunized weekly for 5 months by intraperitoneal injections of 0.5 mg β1 mouse monoclonal IgG1 antibody, which recognizes amino acids 3-6 of human Aβ[2]. Average serum titers were 1:20,000 and 1:4,000 at one and seven days after injections, respectively. Endogenous mouse IgG1 levels in blood were at least ten times higher than the injected amount of β1 antibody. At the end of the experiment mice were overdosed with pentobarbital and brains removed. One hemisphere was immersion fixed for morphological analysis. The other hemisphere was frozen and used for biochemistry.

Histology and Immunohistochemistry

Serial coronal 40μm sections were cut from the formaldehyde-fixed hemispheres. Hematoxylin and eosin (H&E), and Congo red stainings were done according to standard protocols. The Berlin Blue method of Perls was used to visualize ferric iron in hemosiderin[3,4]. Aβ-immunohistochemistry was done with polyclonal antibody NT12 and confirmed with two additional polyclonal antibody to Aβ (1282, courtesy of D. Selkoe; 86/31 courtesy of C. Masters) according to previously published protocols[5,6]. Selected sections were double stained for hemosiderin and Aβ[3,4].

Quantification of Amyloid Load and Cerebral Amyloid Angiopathy

Neocortical amyloid load was estimated on sets of every 20th systematically sampled Aβ–immunostained sections in two-dimensional disectors as previously described[6]. Compact amyloid was defined as Aβ-positive/Congo red positive while diffuse amyloid was defined as Aβ-positive/Congo Red negative[6]. CAA was quantified using a rating scale as used previously in mice3,4 and very similar to that reported in humans[7].

Quantification of Cerebral Hemorrhage

Cerebral hemorrhage is followed by a delayed appearance of hemosiderin-positive microglia cells. Perls' Berlin Blue-stained clusters of hemosiderin-positive microglia were quantified on sets of every 10th systematically sampled sections throughout the entire neocortex as previously

reported[3,4]. Hemorrhage Frequency was obtained by counting the total number of perivascular clusters of hemosiderin positive microglial cells in all sectionsand multiplying the number with the section sampling fraction. Hemorrhage Severity was estimated by grading each hemorrhage counted: Grade 1: Cluster with 1-3 hemosiderinpositive perivascular microglia; Grade 2: Cluster with 4-10 hemosiderinpositive perivascular microglia; Grade 3: Cluster with more than 10 hemosiderin-positive perivascular microglia. The mean grade was taken as severity. Hemorrhage Score was obtained by multiplying hemorrhage frequency with severity providing a general estimate of the extent of microhemorrhage. Additional sets of every 5th section was stained for H&E and screened for remnants of acute intraparenchymal hematomas[3,4]. All quantification was done by three observers (S.B., L.B., and A.S.) and yielded an inter-observer correlation of r=0.71-0.93. The mean was applied for statistical analysis.

ELISA

Human Aβx-40 and Aβx-42 levels were determined by sandwich ELISA from formic acid extracted brain homogenates as previously described[8,9]. Control experiments, in which the β1 antibody was added to Aβ peptide standards, showed that the β1 antibody did not interfere with the ELISA detection of Aβ (unpublished observations).

Coagulation Screening

We have previously reported that thrombolytic treatment enhances hemorrhagic diathesis in APP transgenic mice with CAA[4]. Accordingly, we tested the extrinsic coagulation cascade in the immunized mice by measuring prothrombin times[10]. Briefly, 40μl of platelet-poor, anticoagulated, mouse plasma (EDTA, ~12 mM) was mixed with 40μl of a standard thromboplastin reagent provided by the manusfacturer (recombinant human tissue factor, Innovin, Dade) and with 40μl of 50mM $CaCl_2$. Results revealed that immunized mice had a trend towards shorter prothrombin times compared to PBS-injected control mice (15.4±1.1s vs. 22.6±4.0s; p=0.051). This observation does not support an involvement of the extrinsic coagulation cascade in the increased hemorrhages observed in APP23 mice after Aβ-immunotherapy.

Statistical Analysis

Statistical analysis was done with StatView 5.0.1. Indicated is the mean ±SEM. Significance level was set at $p<0.05$.

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CONCLUSION

The objectives of this thesis have been to provide information about neurogenesis and neurodegeneration in mouse models of aging and AD. Using recently developed morphometric techniques, the impact of aging on the birth of new neurons in B6 mice, and the significance of amyloid deposition on neurodegeneration in APP23 mice was studied in detail. In addition, caloric restriction and immunotherapy, two promising therapeutic approaches in aging and AD research, were applied using these mouse models.

Although much effort has been devoted to relate structural and functional alterations, which occur during normal brain aging and AD, the relationship of these changes to age-related cognitive decline remains debated. While rats have been the animal model of choice for such analyses in the past, more recent aging research has begun to characterize genetically defined mouse strains that exhibit phenotypes relevant to human brain aging. Comparison of brain aging among such inbred mouse strains has then the potential to determine genetic factors related to brain aging and its relation to cognition. Because the B6 line is one of the best characterized mouse strains in neuroscience, and is used as background strain of many genetically-engineered mice, several morphometric parameters have been studied in the aging B6 mice (Calhoun et al., 1998a; Long et al., 1998; Mouton et al., 2002). Interestingly no age-related changes in number of neurons or synapses have been reported in this strain, although the number of dentate gyrus synaptic boutons was correlated to spatial memory performance (Calhoun et al., 1998a), what is consistent with the role of synapses in memory processes. In contrast, it has been suggested that neurogenesis in the adult rodent brain decreases with aging (Seki and Arai, 1995; Kuhn et al., 1996). Given that neurogenesis might be involved in memory and learning (van Praag et al., 1999; Ambrogini et al., 2000; Shors et al., 2001), we have investigated the age-related change in neurogenesis in the aging B6 brain.

Using mice of four age groups, we have found a robust age-dependent decline in the number of newborn neurons in the granular cell layer of the dentate gyrus. Interestingly, the reduction in neurogenesis occurred until late adulthood, with no further decline thereafter. This would indicate a stronger functional importance of neurogenesis in younger mice, with less involvement in aged mice. A decrease in neurogenesis has been suggested to provoke cognitive impairments in

the aging brain (Cameron and McKay, 1999). At least in B6 mice, our results do not support this view, given that B6 mice show no, or only limited, age-related cognitive changes (Calhoun et al., 1998a; Ingram and Jucker, 1999). Our study sets the stage for comparisons between other genetically distinct strains, which do exhibit age-related memory impairments, such as the 129 mouse strain (Ingram and Jucker, 1999).

We have also investigated the influence of long-term caloric restriction (CR) on neurogenesis in the dentate gyrus of B6 mice. Given that CR counteracts age-associated physiological changes in several animal models (Masoro, 2000), an impact of reduced caloric intake could have been expected on neurogenesis. Moreover, short-term CR has recently been reported to enhance neurogenesis in young rodents (Lee et al., 2000; Lee et al., 2002). However, we found no effect of CR on total number of newborn neurons in B6 mice. These differing results might be attributed to the variations in the feeding schedule, to the age of the animals studied, or to the duration of the treatment. Interestingly, we detected a survival promoting effect of CR on glial cells in the hilar region of the dentate gyrus, which might be related to the mild metabolic stress that mice on such a diet are affected with (Masoro, 2000; Mattson, 2000).

A crucial question in AD research is the impact of amyloid plaques and neurofibrillary tangles on neuron loss and cognitive impairments associated with this disease. We have studied the effect of amyloid deposition on neocortical neuron number in APP23 mice. In order to distinguish between the influence of APP overexpression versus amyloid plaques on neurons, mice of two age groups were used: An adult group, at an age where plaques begin to develop, and an aged group exhibiting robust amyloid deposition. Interestingly, estimation of absolute neuron number showed that adult APP mice have a significantly greater number of neurons compared to adult controls. In line with these results, APP and its secreted forms have been implicated in cell growth and survival (Saitoh et al., 1989; Milward et al., 1992; Perez et al., 1997). Whereas wildtype mice did not lose neurons during aging, aged APP23 mice had significantly less neurons when compared to adult APP23 mice. This suggests that neurons are lost in the course of cerebral amyloidosis in APP23 mice. The neurotoxic effect of amyloid deposits was further evidenced by an inverse correlation between total neuron number and plaque load, as well as by an increase in plaque-associated TUNEL-positive neurons in aged APP23 mice when compared to controls. Previous studies of our laboratory have found a more pronounced neuron loss in CA1 of the

hippocampus, a region exhibiting a plaque burden very similar to the neocortex (Calhoun et al., 1998b). This is consistent with reports in the AD brain, where CA1 is heavily affected by neuron loss, whereas the cortex as a whole shows only a moderate reduction in neuron number (Regeur et al., 1994; West et al., 1994). However, robust region-specific neuron loss was observed in subregions of the neocortex such as the entorhinal cortex in AD (Gomez-Isla et al., 1996), and was also observed qualitatively in aged APP23 mice. Studies in other APP transgenic mouse strains did not report neuron losses neither in the hippocampus nor in the neocortex (Irizarry et al., 1997a; Irizarry et al., 1997b; Takeuchi et al., 2000). The reasons for these contrasting results may lie in the use of mouse strains demonstrating a more pronounced presence of diffuse plaques (Irizarry et al., 1997b), or exhibiting a lower total Aβ burden (Irizarry et al., 1997a), or may be due to the use of few animals for quantification, where moderate changes in neuron number could have escaped detection (Takeuchi et al., 2000).

Neuronal death has been suggested to trigger increased addition of new neurons in regions of adult neurogenesis (Gould and Cameron, 1996; Parent et al., 1997). More recently, induction of neurogenesis was also reported in the mammalian neocortex after induced lesions, such as targeted apoptosis (Gu et al., 1999; Magavi et al., 2000). Given that our results demonstrated apoptotic neuronal cell death in the vicinity of plaques in aged APP23 mice, we speculated that neocortical neurogenesis might counteract neocortical neuron loss in these mice. However, we found no evidence for neurogenesis in the neocortex of APP23 mice. In contrast, a severalfold increase in gliogenesis could be detected in aged APP23 mice when compared to controls. A majority of these cells colocalized with microglial markers, and were mostly found in the vicinity of plaques. These results are consistent with an involvement of chronic inflammatory processes, including microglial activation, in AD-related neurodegeneration (Rogers et al., 1996; Akiyama et al., 2000). It is tempting to speculate that neuron loss and gliogenesis may be involved in the cognitive deficits reported in aging APP23 mice (Kelly et al., 2003).

Just a decade ago, very few studies were in progress to test therapeutic strategies for AD. Since then, advances in understanding many of the molecular events leading to neurodegeneration and the genetics of early onset AD have uncovered new drug targets. One treatment strategy has been to target neurotoxic amyloid deposits with anti-Aβ vaccination. Although clinical trials were suspended when several patients developed inflammation in the brain after active

immunization, immunotherapy remains a promising approach to treat AD, when considering the positive results in mouse models of AD and the research on refined approaches.

We passively immunized aged APP23 with an antibody that recognizes amino acids 3-6 of human Aβ. Stereological estimation of amyloid deposition after five months of treatment, revealed a significant reduction in plaque load, when compared to vehicle controls. Although CAA frequency and severity were unaltered, we found an increased number of cerebral hemorrhages associated with amyloid-laden vessels. In a previous study, we have shown that CAA leads to smooth muscle cell loss and to intracerebral hemorrhages (Winkler et al., 2001). It might be suggested that passive immunization further weakens the vessel wall by inducing a local inflammatory response (Maeda et al., 1993) or by loosening vascular Aβ at an ultrastructural level. Given that the majority of AD patients develop CAA in addition to amyloid plaques, the APP23 mouse model is suited for testing future therapeutic paradigms aimed at reducing cerebral amyloidosis.

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