Mechanisms of neuronal loss in aging and Alzheimer's disease

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Mechanisms of Neuronal Loss in Aging and Alzheimer's Disease

Bart P.F. Rutten

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*“(Denn) nicht übermässige Bedenken und lähmende Verzä-
gtheit helfen den Wissenschaften vorwärts und haben ihnen
vorwärts geholfen, sondern ein gesunder Optimismus, der
in froher Zuversicht nach neuen Wegen der Erkenntnis
sucht, da er überzeugt ist, dass sie zu finden sein werden.”*

*„Excessive reservations and paralyzing despondency have
not helped the sciences to advance nor are they helping
them to advance, but a healthy optimism that cheerfully
searches for new ways to understand, as it is convinced
that it will be possible to find them.”*

Dr. Alois Alzheimer

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Chapter 1

General introduction

Mechanisms of Neuronal Loss in Aging and Alzheimer's Disease

INTRODUCTION

Inevitably every one is getting older day by day and year by year. In early life, becoming older is connected with development, maturation and specialization of the brain. The older people become, the higher the risks are of developing age-related diseases and disabilities. As a result, the elderly population is subject to an increased burden of age-related diseases such as cardiovascular disorders, cancer, diabetes, and mental health disorder. Alzheimer's disease (AD) is the most common mental health disorder during aging. AD was once thought to be a rare disorder, but it is now considered a prevalent devastating disease with severe impact on millions of people and their families. Despite the ubiquitous presence and our familiarity with aging and AD, very little is known about the molecular and cellular events underlying neuronal alterations during aging and in AD. So far, there is no available treatment which modifies the molecular and cellular processes involved in AD pathogenesis. To develop rational and effective strategies for prevention or attenuation of neuronal alterations and neuronal loss during aging and in AD, it is crucial to identify the molecular and cellular events that underlie these events. Neuronal loss can be described as loss of neurons or neuronal components and several aspects of neuronal loss (such as loss of neurons or synaptic terminals) can be measured with modern techniques. Animal studies are very useful in the elucidation of molecular and cellular events involved in aging or AD as they allow the investigation of the effects of modifications of molecules *in vivo*. The general aim of my thesis was to scrutinize the roles of various molecular and cellular aspects putatively involved in neuronal loss in aging and AD. This was done by analyzing mouse models of aging and AD with quantitative neuromorphologic, immunohistochemical and molecular techniques. In the first section of this Chapter, background information is provided on the aging process in the brain. Then, the current knowledge of the molecular and cellular events involved in the pathogenesis of AD is described. Finally, current questions and issues on the mechanisms underlying neuronal loss in aging and AD and on potential strategies to attenuate these mechanisms are discussed. On the basis of these questions and issues, I formulated hypotheses which were tested during my research.

THE AGING BRAIN

Epidemiology of aging

Aging is an inevitable process associated with a decline of physiologic function and an increased incidence of disease. Nowadays, approximately 600 million people in the world are 60 years old or more. This huge population of aged individuals is associated with a high incidence and prevalence of age-related diseases. Age-related diseases and disabilities seriously influence the lives of affected people themselves as well as they place a tremendous burden on relatives and on society. Importantly, the population of people older than 60 years is expected to double by 2025 and to reach 2 billion by 2050 (World Health Organization, www.who.int/hpr/ageing/index.htm).

Thus, there is a high need for the elucidation of molecular and cellular events responsible for alterations in the brain during aging with the ultimate aim to prevent age-related diseases and disabilities.

Figure 1. Cellular anatomy of the brain. The brain consists of many distinct brain regions, all having distinct functions which are always dependent on the interaction and connectivity between multiple brain regions. A few brain regions and their names are indicated with arrows in the schematic drawing at the top left of Figure 1. One of the indicated regions is the hippocampus which is a crucial region for learning and memory processes and a region involved in the selectively neurovulnerability in aging and Alzheimer's disease. Every brain region is anatomically characterized by the presence of a huge number of neurons, which communicated with other neurons via certain connections, called synapses. These neurons and synapses work as complicated circuitries involving various neurotransmitters and electrical impulses. Just how they do so is only slowly coming to light. The billions of cells in the brain can be subdivided into various types and subtypes of cells each having a distinct morphology, biology and function. Neurons are the specialized cells of the nervous system responsible for the transmission of electrical impulses to and from the central nervous system. Neurons consist of a cell body, a dendritic tree from which afferent electrical information reaches the cell body and the axon, which is the efferent fiber ensheathed by myelin (from oligodendrocytes) and which transmits electrical signals to the axonal terminal. At the axonal terminal, signal molecules are released into the synaptic cleft which can activate specific receptors located on spines of the dendritic tree of the next neuron. Together, the axonal terminal, synaptic cleft and postsynaptic spine form an entity called the synapse. Many different types of neurons exist in the brain making use of many different signaling molecules. Glia cells are the most numerous types of cells in the brain and can be subdivided into astrocytes, oligodendrocytes and microglia. Astrocytes and microglia cells are activated and recruited to sites of injury. Besides supportive and nourishing, astrocytes have been implicated in formation of synapses, synaptic plasticity and the regulation of the blood-brain-barrier. Similar to astrocytes, microglia cells may become activated in case of injury to the nervous system and can migrate towards sites of injury where they can degrade waste material. Oligodendrocytes form the myelin sheets that encircle and surround the neuronal axons. In doing so, they allow the neurons to transmit signals with an incredible accuracy and speed. Endothelial cells are cells lining the interior of blood vessels. Together with the processes of astrocytes and the pial surface, endothelial cells form the anatomical basis for the blood-brain-barrier allowing only certain molecules to enter the brain. A neuron and its synapse, astrocytes, microglia cells and oligodendrocytes are depicted at the right in Figure 1.

Molecular and cellular events in aging of the brain

Aging is characterized on the cellular level by decrements in maximum cellular function, accumulation of damage and loss of reserve capacity.¹⁻³ These changes are most pronouncedly observed in organs containing post-mitotic cells such as the brain.^{1,4} The brain is considered our most vital organ, responsible for everything from involuntary life support functions to the essence of personality and memory. To understand how the brain works, and what occurs during aging, one has to go to the basics of brain anatomy and brain physiology. Although the knowledge on how the brain works is growing rapidly, we have only limited knowledge of the processes which are responsible for human behavior and its disorders. Some important aspects of neuroanatomy, i.e. several brain regions and cell types, and the unit that transmits signals from one neuron to the other (i.e. the synapse), are depicted in Figure 1. While the brain can age successfully, its cells normally encounter considerable adversities during live. An adversity central to the aging process is the increased production of free radicals which promotes damage to and dysfunction of various metabolic and signaling pathways.^{2,4,5} Free radicals are molecules with a free electron thereby reacting readily with other molecules. Free radicals and radical-derived reactive species are present in all cells and can damage all major cellular constituents at higher concentration.⁶ At lower concentrations, though, they may also play important roles as regulatory mediators in signaling processes.⁷ Most free radicals are derived from oxygen molecules and are often described as oxygen radicals.

The main production sites of free radicals are the oxidative-phosphorylation-protein complexes of the mitochondrial respiratory chain where oxygen radicals are generated as side effect of the production of the “energy molecule” ATP (i.e. adenosine-triphosphate).^{8,9} The way that ATP molecules are generated changes during aging such that more free radicals are produced per ATP molecule.⁸ A description of sources of free radicals and oxygen radicals as well as a description of the molecules involved in detoxification of oxygen radicals, i.e. antioxidants, and their roles in aging is given in more detail in Chapters 3 and 5 of this thesis. Comparative studies between various animals (having different aging rates) have shown that the rate of mitochondrial oxygen radical generation is directly related to the steady-state level of oxidative damage to DNA and is inversely correlated with maximum longevity in higher vertebrates.^{1,8-10} If oxygen radicals are not scavenged by antioxidants, they can damage all macromolecules in cells such as nucleic acids, lipid membranes, and proteins carbohydrates.² Next to an increased production of free radicals, age-related alterations in the mitochondrial respiratory chain may also result in a metabolic compromise,¹¹ which particularly affects neurons as these cells are highly dependent on ATP production. Energy-dependent enzymatic processes may thereby become inefficient in their actions.^{1,6,9} Consequently, biosynthesis of DNA, degradation and resynthesis of proteins,^{12,13} and DNA repair processes^{14,15} may show substantial age-related alterations with for example unrepaired DNA damage^{16,17} as a result. In addition, an appropriate energy supply to cells is necessary for proper maintenance of calcium homeostasis (important for a multitude of cellular functions). Aging is associated with considerable alterations in calcium homeostasis in several types of cells in the brain.^{18,19} These adverse events urge neurons and other cell types of the brain to adapt during aging by increasing their ability to cope with stress, and by remodeling neuronal circuits (Figure 2).^{4,19} Changes in the cellular structure of the brain and the functions of its neuronal circuits are controlled by an array of intercellular

***Figure 2.** Scheme adapted from reference 4 representing a current working frame on some of the most important molecular and cellular events in the aging brain. Aging is characterized by a progressive accumulation of damaged molecules and impaired energy metabolism. Neurons and glia cells may adapt by increasing their ability to cope with stress and may thereby compensate for damaged cellular components. If adaptation is not successful, molecular damage to neurons and inflammatory processes result in synaptic dysfunction or loss, in neuronal degeneration and potentially in neuronal death.*

signaling molecules and intracellular signal transduction pathways. Several of these cellular signal transduction systems show substantial alterations during aging.^{18,19} Examples for the aging central nervous system are gene transcription,²⁰ cellular calcium homeostasis,²¹ protein phosphorylation (alterations in kinases and phosphatases),²² and the response to DNA damage.¹⁶ With respect to the latter example, cells with DNA damage may respond by activating different pathways. They may attempt to repair DNA damage by increasing DNA repair activities, they may inactivate themselves to prevent further damage or they may initiate cell death pathways.¹⁶ Recent evidence suggested that in aging the normal (appropriate) response to genotoxic stress is altered.²³ It was suggested in particular that the apoptosis cell–death pathway is significantly reduced during aging.²⁴ Another major strategy for neurons and other cells in the brain to cope with accumulating damage, is to degrade damaged molecules. Damaged proteins can be degraded into small molecules which can subsequently be used for resynthesis of new proteins.²⁵ The ubiquitin–proteasome pathway in concert with the endolysosomal pathway (both of which are mainly dependent on ATP) are major pathways responsible for recognition and degradation of misfolded, damaged proteins.^{4,26} The efficiency of these pathways however reduces during aging^{12,13,25,27} leading to an increased amount of damaged and misfolded proteins which can accumulate in the form of protein aggregation such as lipofuscin, α -synuclein and others.^{4,26–28} If adaptation to age-related damage is not successful, damaged macromolecules

accumulate in neurons and can elicit an inflammatory response (Figure 2).⁴ Astrocytes and microglia cells are cells mediating the inflammatory response and are recruited to sites of injury and damage.⁴ They may support injured neurons or remove waste material, but can also affect neuronal integrity.⁴ Thus, inflammatory cells are regarded as helpful in normal conditions but can also have toxic effects when overstimulated in certain pathologic conditions.

To complicate the understanding of these matters, the aforementioned age-related molecular and cellular changes affect each other and may create a cascade of degenerating events.^{16,26} In doing so, age-related alterations may ultimately result in dysfunction of synapses and neurons, and to cell death (Figure 2).^{4,26}

A more detailed description of recent advances in understanding the roles of oxidative stress, antioxidants, age-related accumulation of DNA damage and the response to DNA damage in the aging brain can be found in Chapters 3 and 5 of this thesis, and in several recent review articles.^{4,26}

Selective neurovulnerability in the aging brain

The brain is not uniformly affected to the same degree by aging. Selective brain regions and types of neuron have been observed to preferentially harbor age-related alterations (see also Chapter 3). The cerebral cortex and hippocampus are characterized as brain regions severely affected during aging.²⁹ Studies on volumes and metabolism, for example, have indicated that the frontal cortex and medial temporal cortex (wherein the hippocampus is located) are more severely affected than other regions during aging.^{30,31} Molecular studies have furthermore indicated that the cerebral cortex and hippocampus show a higher level of nuclear and mitochondrial DNA damage during aging than other regions.^{16,17,32} In addition, histochemical studies have indicated that protein aggregations and synaptic alterations also preferentially affect types of neuron of the cerebral cortex and hippocampus during aging.^{29,33,34} Traditionally, age-related dysfunction was believed to result from death of neurons during aging. Yet, recent studies, carried out with improved methodologies, have indicated that neurons in the cerebral cortex and hippocampus show no reductions in their total number during aging.^{14,15} Together these findings indicate that age-related dysfunction of cortical and hippocampal regions is connected with local atrophy, decreased metabolism, accumulation of DNA damage, protein aggregations and synaptic alterations and not with age-related loss of neurons. Nevertheless, this does not exclude that types of neuron in other brain regions can be subject to substantial age-related loss of neurons. In this respect, recent studies have suggested a so far unknown inverse correlation between types of neurons showing an age-related decline in the rate of nDNA repair and types of neurons showing a reduction in number of neurons during aging (see Chapter 3 for further details).^{14,15} The basis and relevance of this aspect as well as of other aspects involved in the selective neurovulnerability during aging are currently unclear. Nonetheless, one may argue that the alterations occurring during normal aging might set the stage for dysfunction of the brain during aging and for the pathogenesis of neurodegenerative disorders that may be triggered by particular genetic predispositions or environmental factors.^{4,26,34}

Strategies to attenuate age-related neurodegeneration

Several strategies have been employed to attenuate age-related neurodegenerative processes in the brain. Although most of them have not been successful so far, some strategies have appeared to show a substantial impact on age-related neurodegeneration.

Antioxidants

Antioxidants are molecules characterized by their ability to scavenge and neutralize free radicals.^{6,35,36} More than ten thousand compounds are known to have antioxidant properties. Endogenous antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase are produced by cells themselves and are enzymatic.³⁶ Exogenous antioxidants on the other hand, are non-enzymatic, and can be taken up into the body via the diet.³⁶ In Chapter 5 of this thesis, the actions and effects of antioxidants during the aging process are described in more detail. Attenuation of age-related oxidative damage and degenerative events may be achieved by upregulation of the natural defense to oxygen radicals, i.e. to increase the amounts or activities of antioxidants.^{4,37,38} In line with this way of thinking are reports in the literature showing that depletion from normal amounts of antioxidants can result in a reduced life span, accelerated age-related changes and enhanced injury.³⁹⁻⁴² Furthermore, overexpression or supplementation of antioxidants has been reported to attenuate age-related and neurodegenerative changes in mammals and to extend life span in lower organisms.⁴²⁻⁴⁹ However, other studies suggest that the correlation between upregulating antioxidants and attenuating age-related changes and extending life span, may not be that simple.^{46,50,51} One drawback of some of these studies is that they were carried out on animal species (e.g. yeast, roundworms and fruitflies) which are distant from humans in a phylogenetically and evolutionary perspective. In this respect, mammalian species offer better, more physiologic models to study the effects of antioxidants on the aging process in the brain. The number of studies carried out to assess the effects of antioxidants on the aging brain is however limited. One study reported that mice overexpressing normal human Cu/Zn superoxide dismutase (the isoform mainly active in the cellular cytoplasm) had reduced oxidative damage in the central nervous system at high age as compared with control mice.^{44,52} Another study showed that a diet enriched in polyphenols, i.e. natural antioxidants, decreased age-related changes in the central nervous system and in behavior in rats.⁵³ Furthermore, according to a recent study dietary antioxidants may ameliorate age-related disorders of cognitive functions in dogs.⁵⁴ Together these studies indicate that the use of antioxidants can decrease age-related damage and dysfunction in the brain. Although it seems attractive to simultaneously upregulate endogenous and exogenous antioxidants as tools to attenuate age-related free-radical induced damage, it remains to be established whether the combination of both endogenous and exogenous antioxidants results in pronounced attenuation of age-related changes in those regions and types of neuron in the mammalian central nervous system most affected during aging.

Caloric restriction

Although it was already found in 1935 that dietary caloric restriction (CR) prolongs mean and maximum life span,⁵⁵ it was not until the 1970's and 1980's that these findings were confirmed

in studies reporting that restriction of dietary caloric intake resulted in an extension of maximum life span by as much as 50% in roundworms and rodents and attenuated the risk of the most prominent age-related diseases such as cardiovascular disease, cancer and diabetes in rodents.^{56,57} Up to now, dietary restriction of calories is the only intervention strategy which has consistently been shown to extend life span and to attenuate age-related degenerative events in several animal species.^{58,59} The exact molecular and cellular events underlying these effects however remain largely elusive. So far, gene expression studies have indicated that CR suppresses several signaling pathways which are normally upregulated in the aging process.²⁰ Some of the molecules involved in these pathways are proteins expressed in response to oxidative stress⁵⁹, proteins involved in energy metabolism,^{18,58,59} proteins involved in regulation of the calcium homeostasis¹⁹ and molecules involved in the immune response.¹⁹ CR has furthermore been shown to stabilize mitochondrial function associated with a decreased oxygen radical production (mainly at respiratory chain complex I),¹ to stimulate DNA repair⁶⁰ and to enhance the expression of other genes involved in neuronal survival and plasticity such as protein chaperones and neurotrophic factors.⁴ Interestingly, some reports have indicated that caloric restriction is associated with a reduced number of synapses⁶¹ in cerebral cortex and retinal ganglion cells,⁶² i.e. alterations which – at first glance – do not seem beneficial to the brain. Thus, although almost all studies point to a beneficial action of CR on the aging process in the brain, the current knowledge on the underlying molecular and cellular mechanisms is still very limited. It is also unknown so far whether the actions of CR show a regional or cell type specificity in the brain, as it is plausible that signaling pathways in distinct regions or cell types respond differently to CR.

Other strategies

An increasing number of reports in the literature indicate that physical and mental activities are positively related with cognitive functions at older age.^{63,64} Animal studies are in line with the findings of beneficial actions of exercise and point to induction of neurorestorative and neuroplastic changes as an underlying mechanism.⁵ Besides these hopeful reports, a number of observational studies of postmenopausal women have indicated that estrogen replacement therapy may protect against age-associated memory decline in non-demented women.^{65–67} Recent findings in animal models point to enhanced synaptic plasticity as the responsible cellular event induced by estrogen supplementation.⁶⁸ However, further studies are necessary to pinpoint the mechanism(s) responsible for the observed enhanced synaptic plasticity and prospective studies with large cohorts should be implemented before actual conclusions can be made in this respect.

ALZHEIMER'S DISEASE

Historical perspective

The German neuropsychiatrist Alois Alzheimer was the first to describe a patient displaying several cardinal symptoms of a disease which was later to bear Alzheimer's name. Alzheimer described the relatively young woman Auguste Deder who suffered progressively from

symptoms of cognitive impairments, problems with reading and writing and a gradual loss of other higher mental functions. After Auguste Deder's death in 1906, Alzheimer examined her brain and described a thinner cerebral cortex than normal and two other abnormalities: senile plaques and neurofibrillary tangles. Alzheimer defined the clinicopathological syndrome of Auguste Deder as 'presenile dementia' and presented her case in 1906 on a conference in Munich and published his findings in 1907.⁶⁹ After this first description, more cases with similar symptomatology were recognized and described by Alzheimer and others. After several reports of cases with the same symptomatology, Kraepelin (who was the head of the Munich clinic where Alzheimer worked at that time) initiated the definition of a syndrome and proposed to attach Alzheimer's name to the disease.⁷⁰ For decades after Alzheimer's first description, little progress was made in defining and elucidating the pathogenesis of AD, although there was growing awareness of the commonness of the disease. In the 1960's the development of electron microscopy allowed for the first time to describe the characteristic neuropathologic lesions (i.e. senile plaques and neurofibrillary tangles) in the brains of AD patients on the ultrastructural level. The use of neurochemical techniques, which became available in the 1970's, allowed for the first definition of neurochemical changes in AD. These observations led to the development of the first pharmacological agents for AD which focused on the restoration of a certain neurotransmitter, acetylcholine. These agents are currently among the few medications which are proven to be effective in decreasing the progression of some of cognitive symptoms in AD. However, research carried out in the 1980's and 1990's indicated that several – if not all – neurotransmitters show deficits and alterations in AD. It is now well known that the neuropathology of AD is characterized by the presence of senile plaques and neurofibrillary tangles, but also by neuron and synaptic loss in selective regions (but involving various neurotransmitter systems). These findings have prompted researchers towards further investigations of the molecular and cellular mechanism underlying and preceding the formation of senile plaques and neurofibrillary tangles and their connections with neuronal and synaptic loss. It has been observed that neuritic plaques mainly consist of the β -amyloid protein ($A\beta$) and neurofibrillary tangles of hyperphosphorylated tau protein. In parallel to these two classical hallmarks, a hot debate emerged between researchers favoring different theories on the pathogenesis of AD. Some scientists ("Tauists") favored the idea that hyperphosphorylation of tau protein is the key event whereas others ("Baptists") favored the production and accumulation of $A\beta$ as the key event in the cascade of AD pathophysiology.⁷¹ The finding by scientists in the 1990's that mutations in the genes of presenilin-1, presenilin-2, and amyloid precursor protein are responsible for early onset-familial cases of AD and lead to enhanced generation of $A\beta$ protein bolstered the "baptistic" idea that $A\beta$ containing plaques were the crucial aspects of AD pathogenesis (reviewed in ref. 72). Other studies indicated however that the presence of neurofibrillary tangles in certain brain regions correlated better with the clinical symptoms of AD patients than any other neuropathologic correlate (including plaques).⁷³ In addition to these findings, another genetic variant, the ApoE4 genotype was found to be a main genetic risk factor for AD. Over the last years, considerable progress in neuropathological, neurochemical, and molecular studies on AD has been made resulting in the emersion of a rough outline of the disease cascade. This progress and the related efforts to identify targets for therapeutic intervention have been markedly assisted by the development of cellular and animal models.

Diagnosis of Alzheimer's disease

Dementia, a syndrome characterized by deteriorating cognitive functions with significant impact on patients' daily life, is one of the most common age-related mental health problems.⁷⁴ Dementia is commonly recognized by physicians with use of the criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition.^{75,76} AD is the most important cause of dementia. AD is a progressive neuropsychiatric disorder clinically characterized by a decline in memory, thinking, comprehension, calculation, language, learning capacity and judgment.⁷⁴ The diagnosis of AD is commonly based on the criteria developed by the National Institute of Neurologic and Communicative Disorders and Stroke-AD and Related Disorders Association,⁷⁷ according to which the diagnosis is classified as definite (i.e. clinical diagnosis with *postmortem* histologic confirmation), probable (i.e., typical clinical syndrome without histologic confirmation), or possible (i.e. atypical clinical features but no alternative diagnosis apparent; no histologic confirmation). According to the age of onset (and genetic predisposition), AD can be manifested in two forms:⁷⁸

1. Familial – early onset – form of AD consisting of patients harboring autosomal dominant mutations. Symptoms may begin at an early age (as early as thirty years) and approximately 3–5% of all AD cases are of this familial form.⁷⁸
2. Sporadic – late onset form – of AD in which no single genetic mutation is identified in these patients to be responsible for the disease. Symptoms generally begin from an age of 65.⁷⁸

Epidemiology of Alzheimer's disease

The number of people with AD in the Netherlands currently stands at around 125,000.⁷⁹ With every five years of age, the percentage of persons with AD increases by a factor two.^{74,80} The influence of age on the prevalence of AD is underscored by studies reporting that a very low percentage (<1%) of people under an age of 65 have AD, whereas 10–30% of people aged 85 and more have AD (depending on the inclusion criteria).^{81–83} Due to the increased number of elderly people in the general population, these numbers will substantially increase over the next decades,⁷⁹ and if curative treatments fail to emerge, the number of people with AD in the Netherlands will be approximately 150,000 by 2010 and 290,000 by 2050,⁷⁹ while the number of people with AD in Europe has been estimated to reach 20 million by 2050.⁸⁴

The annual incidence rate of AD is approximately 1% in people aged 65 to 70 years and increases to approximately 6–8% in people over age 85.⁷⁴ The incidence rates of AD are slightly higher for women and for people of certain races.^{80,85} The duration of AD varies considerably from 2 to 20 years,⁸⁵ although the median survival time for AD patients has been estimated to be 3 to 4 years.⁸⁶ The risk of developing AD or another dementia at some point during life depends on the incidence rate and life expectancy from birth and has thus been found to be considerably higher in women (12 and 19%) than in men (6 to 10%).⁸⁷ The cost of caring for patients with AD is enormous and has been estimated at approximately € 100,000 per patient per 10 years.⁸⁸

Together these figures designate that AD places a very serious burden on general society which will be even greater in the near future.

Genes and genetic risk of AD

Mutations in three genes have been found in patients with the familial form of AD: mutations in the genes for amyloid precursor protein (APP) on chromosome 21,⁸⁹⁻⁹¹ presenilin 1 (PS1) on chromosome 14⁹²⁻⁹⁴ and presenilin 2 (PS2) on chromosome 1.^{95,96} Of these three genetic mutations, mutations in PS1 are responsible for most cases of familial AD.⁹⁰ Together, these three mutations only account for a small proportion of AD patients (approximately 3–5%).⁷⁸

An allelic variant of another gene, the ApoE4 genotype,⁹⁷ has been found to be associated with both familial and sporadic AD.⁹⁸⁻¹⁰⁰ People with a single ApoE4 allele have a twofold increased risk of AD, whereas the risk is fivefold increased in people with two ApoE4 alleles. The proportions of AD related to the ApoE4 genotype is approximately 20%.¹⁰¹ Additional putative loci for genetic variants or mutations have been identified on chromosomes 12p12 and 10q24.⁸⁵

Risk modifiers of AD

Several environmental, genetic and life style factors are known to influence the risk of AD.⁸⁵ The risk of AD has been found to be significantly higher among poorly-educated people than among well-educated people,^{102,103} and significantly lower in people raised in a nurturing and supportive environment than people not raised under these circumstances.^{103,104} Other recent studies suggest that people engaged in physical or mental activities during late life, preferably complex or dynamic activities, have a lower risk of AD.^{63,64} Traumatic head injury earlier in life is connected to an increased risk of AD.¹⁰⁵ Furthermore, a large proportion of patients with Down syndrome (trisomy of chromosome 21) develops AD symptoms already at an early age.¹⁰⁶ The risk of AD is also two to three-fold increased in people with a positive family history for Down syndrome.^{107,108} Dietary intake of natural or supplementary antioxidants,¹⁰⁹⁻¹¹¹ the use of anti-inflammatory agents^{112,113}, and the use of lipid-lowering agents or statins¹¹⁴⁻¹¹⁶ have recently been associated with a decreased risk of AD. Use of estrogen by postmenopausal women has furthermore been associated with a lower risk of AD.⁶⁵⁻⁶⁷ In this respect, however, two clinical trials of estrogen replacement in women with AD failed to show a significant benefit.^{117,118} In people with hyperlipidemia, diabetes or hypertension, the risk of AD is increased.^{119,120} Moderate alcohol consumption however has recently been associated with a reduced risk of AD.¹²¹ Prospective studies with large cohorts have furthermore shown that smokers have a two-to fourfold increased risk of AD, with a particular increased risk among smokers harboring the ApoE4-genotype.^{122,123}

Neuropathology of Alzheimer's Disease

The classical hallmarks in the neuropathology of AD are the neuritic plaques and neurofibrillary tangles (Figure 3).¹²⁴ AD neuropathology is additionally characterized by the presence of diffuse plaques, atrophy of the brain and loss of neurons and synapses.¹²⁴

Neuritic plaques

Neuritic plaques are microscopic foci of extracellular A β deposition associated with and surrounded by injured axons and dendrites.¹²⁵ They are preferentially found in limbic and other association cortices and in hippocampus. Neuritic plaques contain extracellular deposits of A β in a filamentous, star-shaped masses of A β fibrils (Figure 3A).¹²⁵ Their cores are detectable by Congo red or Thioflavin S histochemistry. Plaques usually contain both A β 40 and A β 42 although most of the fibrillar A β found in neuritic plaques is composed of A β 42, the more hydrophobic form and particularly prone to aggregation.^{72,126} The injured or dystrophic neurites are often dilated and tortuous and are marked by ultrastructural abnormalities that include enlarged lysosomes, numerous mitochondria, and paired helical filaments.¹²⁵ Neuritic plaques are also intimately associated and surrounded by activated microglia and reactive astrocytes which display abundant glial filaments.¹²⁷ Microglia cells are usually located within and adjacent to the central amyloid core of the neuritic plaque, whereas astrocytes often ring the outside of the plaque, with some of their processes extending towards the core of the plaque. The time in which such a neuritic plaque develops in AD is unknown, but these lesions probably evolve gradually over a substantial period of time.⁷²

Diffuse plaques

Diffuse plaques are aggregations of A β with relatively light, amorphous A β immunoreactivity without a clearly fibrillar core. Diffuse plaques are found in limbic and association cortices but also in many brain regions not clearly implicated in the typical symptomatology of AD.¹²⁵ They mainly consist of A β 42 species,^{128–130} appear to be associated with little or no detectable neuritic dystrophy,⁷² and represent most likely precursor lesions of neuritic plaques.⁷² Healthy aged humans free of AD often show diffuse plaques in limbic and association cortices, the same regions where AD patients show mixtures of diffuse and neuritic plaques.⁷²

Amyloid angiopathy

Small arterioles, venules, and capillaries within the cortex of AD patients frequently bear small amyloid deposits (Figure 3A).¹³¹ The A β species principally involved in microvascular angiopathy is A β 40, although the initial depositions may contain A β 42.¹³² The extent of amyloid

angiopathy varies widely among AD brains that have relatively similar burdens of parenchymal (i.e., plaque associated) A β ,⁷² and the contribution of microvascular amyloidosis to cortical dysfunction occurring in AD remains largely unclear.¹³³ Vessels with A β can furthermore be observed in the brains of elderly subjects without AD, a condition called congophilic amyloid angiopathy.¹³⁴ Vessels with A β deposits may rupture and may thus lead to cerebral hemorrhages,¹³³ although the large majority of AD patients do not experience cerebral hemorrhages.

Neurofibrillary tangles

Neurofibrillary tangles (NFT) are large bundles of abnormal fibers of hyperphosphorylated tau protein. Tau protein is a microtubule-associated protein with a supportive function in the cell's cytoskeleton and is involved in axonal transport. The gene of tau protein is located on chromosome 17 and mutations in this gene account for a rare disease called frontotemporal dementia with parkinsonism.¹³⁵ A variety of kinases have been shown to be capable of phosphorylating tau *in vitro*.¹³⁶ Nevertheless, it has not become clear which kinases are principally responsible for initiating the hyperphosphorylation of tau *in vivo* eventually leading to its dissociation from microtubules and aggregation into insoluble paired helical filaments.⁷² NFT are normally located intraneuronally although they can also be found in the extracellular space as so called 'ghost tangles', which are presumably NFT of neurons that have died.^{137,138} Electron microscopy has indicated that most of the NFT-fibers consist of pairs of 10-nm filaments wound into helices, called paired helical filaments (PHF) and that NFT-fibres are not bound to membranes.¹³⁷ Notably, NFT are mostly formed in large projection neurons having long axons.^{138,139} Proper function of these projection neurons depends on a strong cytoskeleton, a well regulated axonal transport system and a well regulated energy supply.

Neuron loss

Neuron loss has classically been considered as a key feature in AD neuropathology. The question whether neurons are lost in a certain brain region is however not easily answered for two important reasons. First, the technologies currently available do not allow the investigation of the number of neurons in the same individual for more than one time during the individual's live. Because microscopic resolution is necessary to address numbers of neurons, scientists can currently only achieve this resolution by investigating *post mortem* brain tissue. Usually the results of these analyses are then grouped according to the age or disease state of the individuals analyzed and subsequently compared. Thus this strategy cannot fully answer whether the same individual has lost neurons during aging. However, this approach has been adapted by many investigators to address research questions concerning reductions in neuron numbers. A second problem in answering this 'neuron loss' question is that, up to recently, scientists have been using biased methods to quantify the numbers of neurons within a region of interest.^{140,141} In this respect, recent developments in quantitative neuromorphology have resulted into better methods allowing high precision estimations of total numbers of neurons by using stereologic quantification techniques.^{140,141} However, these modern stereologic methods have not been applied extensively on AD brains yet. The few stereologic studies on AD carried out so far though, have indicated substantial loss of neurons within area CA1 of the hippocampus,^{73,142-145}

Figure 3. Photomicrographs of two classical hallmarks of AD neuropathology. *A* depicts β -amyloid containing plaques (arrows indicate a few neuritic plaques of various size and the arrowhead indicates a vessel with amyloid angiopathy) and *B* depicts neurons with neurofibrillary tangles. Scale bar represents 150 μ m in *A* and 20 μ m in *B*.

in prefrontal cortex (Brodmann's area 9)^{73,138,146} and in entorhinal cortex of AD patients as compared to age-matched controls.^{145,147,148} However, no significant loss of neurons was observed within area CA2/3 of the hippocampus of AD patients.¹⁴⁴ It was furthermore found that loss of neurons within area CA1 correlated well with the level of AD neuropathology staging according to the Braak staging¹⁴⁹ and with cognitive status as tested with the Mini Mental State Examination.⁷³ Thus, these stereologic studies confirm that AD is characterized by substantial neuron loss in selective brain regions. Nevertheless, there is still a high demand for additional stereologic studies to further scrutinize the selective neurovulnerability in AD brain, to describe temporal and spatial features of neuron loss and to compare these with the temporal and spatial development of other neuropathologic hallmarks. In this respect, some aspects have already been elucidated. For example, recent studies indicated that cognitive deficits in AD were closely reflected by NFT and loss of neurons in hippocampus and frontal cortex in AD, and not by the burden of extracellular neuritic plaques.⁷³ Furthermore, it has been demonstrated that neuronal populations that differ in proneness to NFT formation are lost at different rates in AD, and that nonphosphorylated neurofilament protein-enriched neurons emerge as a strikingly vulnerable subpopulation of neurons.^{138,146,147} As these neurons are crucial in furnishing key subsets of corticocortical connections, their preferential loss indicates that neurons providing connections between association cortices and the hippocampus are at high risk for degeneration in AD.^{34,73}

Finally, it should be mentioned that shrinkage of neuronal cell bodies and reductions in the dendritic trees of neurons are prevalent neuronal abnormalities which may precede and accompany frank neuronal loss.^{150–152}

Synapse loss

A multitude of evidence in the literature has converged and pinpoints to synaptic failure and synaptic loss as key events responsible for cognitive decline in AD patients (reviewed in ref. 153–155). It is noteworthy that studies have found loss of synapses, for example as measured by densities and numbers of synaptophysin-immunoreactive-presynaptic-boutons, to be

substantially greater than loss of neurons in AD.¹⁵⁶ Similar to the pattern of neuronal loss in AD, selective regions in brains of AD patients show the most pronounced synaptic alterations. These regions are the prefrontal cortex, the hippocampal stratum moleculare receiving inputs from the entorhinal cortex, and the stratum radiatum of hippocampal area CA1^{29,34} Interestingly, these same regions are also known to show alterations in synaptic content and function during aging, albeit much less pronounced than in AD.^{157–159} The exact mechanisms underlying the synaptic alterations and synaptic loss in AD remain however elusive.

Thus, the neuropathology of AD is characterized by A β containing plaques, hyperphosphorylated tau containing NFT, loss of neurons and loss of synapses. All of these hallmarks have a certain, partially overlapping, regional selectivity. To describe the temporal and regional development of NFT and neuritic plaques, neuropathologists have formulated certain staging schemes. Two of the most validated and used schemes for staging the neuropathology of AD are the Braak staging¹⁶⁰ and the CERAD staging.¹⁶¹ The CERAD staging is based on (semi)quantitative assessment of neuritic plaques (CERAD), plaques and NFT in neocortex and hippocampus,¹⁶¹ whereas the Braak staging is mainly based on the hierarchic spreading of NFT.¹⁶⁰

Treatment of AD

The current standard of medical care for mild to moderate AD includes treatment with acetylcholinesterase inhibitors and with an N-methyl-D-aspartate receptor antagonist, memantine (which has recently been approved for the treatment of advanced AD in the United States).⁷⁴ These drugs however have only modest symptomatic effects and are of limited benefit to most patients.⁷⁴ Some medication is available for mood disorders, agitation or psychosis which can occur in late stages of the disease. Most importantly, however, no treatment with a strong disease-modifying effect is currently available.^{74,162}

In summary, the growing aging population and the concomitant increasing incidence of various age-related diseases and disabilities place an enormous burden on society. To develop preventative and therapeutic strategies, it is crucial to identify the molecular and cellular mechanisms underlying aging and age-related disorders. AD is the most prevalent age-related mental health disorder with an enormous impact on individual patients, their families and society. Unfortunately, there is currently no efficient treatment strategy for AD at hand. The elucidation of the pathogenetic cascade of AD is therefore of utmost importance with the ultimate goal to develop effective preventative and therapeutic strategies to combat this devastating disease.

The A β cascade hypothesis of AD pathogenesis

Several hypotheses on the pathogenetic cascade of AD have been formulated and none of these is without any controversy. The genetic evidence that familial cases of AD are caused by mutations in presenilins and in APP, and the biochemical evidence that these mutations lead to an enhanced generation of A β protein have led to the proposal of the A β cascade hypothesis of AD pathogenesis.^{72,163,164} In this respect, cellular and animal models have been and are still very useful in determining the functions and molecular characteristics of factors putatively involved in AD pathogenesis.¹⁶⁵

Amyloid precursor protein and its processing to A β

APP is a member of the family of Amyloid Precursor-Like Proteins (APLP).^{166,167} The APLP family comprises polypeptides with molecular weights varying between 110 and 140 kDa which are ubiquitously expressed. The main members of this family are APP, APLP1 and APLP2. The molecules can have various molecular weights as a result of alternative splicing and a variety of posttranslational modifications.^{168–171} Although APP has mainly been discussed in the literature as the source of A β , it has become evident over the last years that APP and its fragments have roles in normal physiology. Mice genetically engineered to lack both APP and APLP2 or both APLP1 and APLP2 die during late embryogenesis or shortly after birth while mice genetically engineered to be only deficient in either APP, APLP1, or APLP2 are viable and have only minor neurological deficits.¹⁷² APLP2 seems to have a critical role in these developmental problems: mice lacking APP and APLP1 are viable whereas mice lacking APP or APLP1 in combination with a lack of APLP2 die early postnatally.¹⁷³ Furthermore, mice lacking all three APP genes have been reported to be subject to a high incidence of cortical dysplasia including excencephaly, polymicrogyria, and neuronal migration defects.¹⁷⁴ These findings suggest an important role for the APLP gene family in neurodevelopment and neuronal migration.¹⁷⁵ Besides this, APP has been proposed to play neurotrophic and neuroprotective roles. This is based on findings that the level of APP protein increased in neurons after various forms of injury to the central nervous system,^{176–178} and that various strains of APP overexpressing mice have shown enhanced synaptophysin immunoreactivity.^{176,179,180} Mice lacking APP showed furthermore substantial disturbances in long term potentiation in hippocampal area CA1 when investigated at higher ages (i.e. 12 and 24 months of age).¹⁸¹ APP is a single transmembrane polypeptide that is translocated into the endoplasmic reticulum during translation.¹⁷⁰ Posttranslationally modifications

Figure 4. Scheme of the two main pathways responsible for processing of Amyloid Precursor Protein. APP is a transmembrane protein with its C-terminal site located within the cytoplasm and the N-terminal site in the extracellular space or in the lumen of vesicles belonging to the secretory system. A depicts pathway A in which APP is cleaved by α -secretase (carried out by ADAM10) into α -APP and C83. Subsequent cleavage of C83 by γ -secretase (carried out by a multiprotein complex with PS1 and PS2 involved) can then release the P3 and P6 fragments. B depicts pathway B in which APP is cleaved by β -secretase (carried out by BACE-1) into β -APP and C99. Subsequent cleavage of C99 by γ -secretase can then release the A β molecule and P6 fragment. Although also occurring under normal conditions, pathway B has been implicated to have the overhand in the pathogenesis of AD.

occur in the secretory system of the cell where APP molecules "mature".¹⁷⁰ The half-life of APP is relatively brief (approximately 1 hour in most cell types).¹⁷⁰ APP can undergo a variety of proteolytic cleavages thereby releasing derivatives into vesicle lumens (of organelles) and into extracellular space (Figure 4). These processes occur predominantly during and after the trafficking of APP through the secretory system. The first proteolytic cleavage is made by the α -secretase molecule and occurs 12 amino acids N-terminal to the single transmembrane domain of APP.^{182,183} Recent studies have shown that certain members of the metalloprotease family, i.e. the so called ADAM proteins, are responsible for α -secretase cleavage.¹⁸⁴ APP cleavage by α -secretase results in the release of the large soluble ectodomain fragment (α -APPs) into the lumen/extracellular space and retention of an 83-residue C-terminal fragment (C83) in the membrane (Figure 4A).¹⁸⁴ Alternatively to α -secretase cleavage, APP molecules can be cleaved by β -secretase generating a slightly smaller ectodomain fragment (β -APPs) and retaining a 99-residue C-terminal fragment (C99) in the membrane (Figure 4B).¹⁸⁵ Cleavage by β -secretase, is performed by the recently identified protein called BACE-1.^{186,187} This cleavage can be followed by cleavage at the C-terminus of the A β region by a multi-protein complex called γ -secretase (Figure 4B). Presenilin-1 and presenilin-2, are two out of many proteins forming this complex.¹⁸⁸⁻¹⁹⁰ Cleavage of C99 by γ -secretase generates the A β peptide (either A β 40 or A β 42) and a fragment called P6 (Figure 4B). Although α -secretase cleavage normally has the overhand, both pathways of APP processing (Figure 4A and B) can occur in physiologic conditions.¹⁹¹ In line with this, A β has been detected in both cerebrospinal fluid and plasma in healthy subjects throughout life.¹⁹² In addition to the generation of A β , the sequential actions of the α - and γ -secretases produce a fragment called P3 (Figure 4A).¹⁹³ Precisely where during its intracellular trafficking APP is cleaved by α -, β -, and γ -secretase cleavages is not settled yet.⁷² It is known that α -APPs can be generated at the plasma membrane¹⁹⁴ and in vesicles of the late compartment of the cellular secretory systems.¹⁹⁵ The β -secretase cleavage step can occur in the late compartment of the cellular secretory systems.¹⁹⁶ The sites of γ -secretase cleavage are not entirely clear so far.¹⁹⁷ Accumulating evidence suggests that A β 40 and A β 42 are mainly generated during the internalization and endosomal processing of APP.^{198,199} It is still elusive whether A β 42 is generated early in the secretory trafficking of APP or principally after APP has reached the cell surface.⁷² Evidence suggests that secretase-cleavages of APP occur at or near the cell surface, perhaps in substantial part in recycling endosomes.^{188,199} APP can be anterogradely transported by fast axonal transport systems²⁰⁰ and is present in axonal terminals.²⁰¹ Other recent studies have reported that APP can also be transported retrogradely towards the cell body and to somatodendritic surfaces.²⁰² APP is abundantly expressed in neurons which can secrete substantial amounts of A β .¹⁹¹ Besides neurons, other cells types (astrocytes, microglia, and endothelial and smooth muscle cells) express APP and can release A β .¹⁹¹ Once formed, A β peptides can be catabolized by proteases, some of which have recently been identified to be neprilysin, insulin-degrading enzyme, and angiotensin-converting enzyme.^{203,204} Thus, evidence accumulates for a physiological role of APP in development, neuronal migration, injury response and synaptic action.^{178,205} The exact functions and molecular targets of APP and the various fragments after secretase cleavages of APP remain largely unclear.

Figure 5. Scheme adapted from reference 72 depicting a sequence of pathogenetic steps of familial forms of Alzheimer's disease according to the Aβ cascade hypothesis.

Figure 6. Scheme summarizing the major candidates for age-related loss of neurons and synapses in AD according to the A β cascade hypothesis. These candidates are mutant APP, mutant PS1, the C99 fragment of APP, intraneuronal A β , extracellular oligomeric A β , and extracellular fibrillar A β . The dashed lines represent undetermined associations. Note that PS1 mediates γ -secretase cleavage of the C99 fragment of APP into A β (thin-lined arrow).

Presenilins

Presenilins (PS) are integral multipass membrane proteins responsible for regulated intramembrane proteolysis of various proteins such as APP, Notch 1, ErbB-4, and E-cadherin.^{189,206} PS1 and PS2 are expressed throughout the brain and their genes are located on chromosome 1 and 14, respectively. Presenilins undergo constitutive endoproteolysis into fragments within endoplasmic reticulum vesicles and undergo stabilization in the Golgi apparatus.²⁰⁷ Once formed, multiple presenilin fragments can associate into higher molecular mass complexes. Mice genetically engineered to lack presenilins die early during development and display defects in neurogenesis and neuronal differentiation.²⁰⁸ These effects are mainly a result of defective Notch signaling.²⁰⁸ When the lethal embryonic effect of the PS1 deficit was circumvented by a conditional knockout approach, loss Cajal-Retzius cells in the neocortex and associated disturbances in cortical lamination and radial glial development were observed in live born animals.²⁰⁹ These findings indicate that PS1 is critically involved in neurodevelopment. As mentioned before, mutations in PS1 and PS2 cause early-onset cases of AD. Mice expressing the same mutations in presenilins as familial AD patients are born viable without major abnormalities but do show disturbances of calcium homeostasis, have increased levels of oxidative stress,^{210,211} and increased levels of A β 42 and A β 43.²¹² The increased generation of A β is mediated by a mutation-induced gain-of-function of the γ -secretase cleavage of APP. Controversy exists whether the presenilins participate directly in γ -secretase cleavage or whether they play an indirect role via membrane trafficking.^{188,189} In summary, presenilins are involved in several important molecular signaling pathways and play important roles in normal development and physiology.^{213,214}

The cascade of events

The A β cascade hypothesis poses that A β is the key molecule in the pathogenesis of AD and that aggregation of A β leads to oxidative injury, dysfunction and loss of synapses and neurons, the formation of neurofibrillary tangles, as well as to an inflammatory response (Figure 5).^{72,162,164,215} Exactly how A β protein or altered processing of APP would result into the entire neurodegenerative cascade is a matter of intense research and scientific debate (for review see^{164,216}). The main issue herein is which molecule(s) in the entire A β cascade is (are) responsible for dysfunction and death of neurons and synapses.¹⁵⁴ According to the original A β cascade hypothesis, extracellular aggregations of fibrillar A β would be responsible for neuronal dysfunction and loss. Several reports in the literature have suggested that extracellular aggregations of fibrillar A β are responsible for neurodegeneration and death of neurons,²¹⁷ raising the theory that the formation of extracellular A β 40/42 aggregations form the crucial event in AD. Studies on human and animal brains have indeed provided evidence that extracellular neuritic plaques are associated with a substantial activation of microglial cells^{218,196} and astrocytes.^{219,220} Neuritic plaques have furthermore been shown to affect surrounding neurites *in vivo*,^{221,222} and fibrillar A β aggregates have been shown to display neurotoxic effects *in vitro*.^{223,224} Nonetheless, it has remained unclear whether A β -containing neuritic plaques are actually associated with neuronal cell death *in vivo*. Several studies with APP transgenic mice, which have abundant age-related plaque formation, have not led to convincing evidence for plaque-associated neurotoxicity.^{180,216}

On the basis of recent studies which suggested that oligomeric extracellular species of A β protein, rather than the fibrillar forms of A β , induced neurotoxicity *in vitro*,²²⁵ focus has shifted towards oligomeric forms of A β as being the culprit in neurotoxicity.^{72,155} Oligomeric A β species are complexes of a small number of A β peptides. These complexes are mainly soluble and have been shown to induce various synaptic alterations.¹⁵⁵ They can exist both extra- and intracellularly and are difficult to detect with immunohistochemical techniques. Again, actual prove that soluble oligomeric species of A β are connected to neuron loss *in vivo* is not at hand. Given that A β peptides are generated intracellularly (e.g. in plasma membranes and in membranes of the secretory/endosomal system) scientists have attempted to identify A β species within neurons, which turned out to be rather difficult. Very recently, A β species have been detected within neurons in the brains of AD patients and not in controls,²²⁶ pointing to a potentially important role for intraneuronal species of A β .

Besides the putative neurotoxic properties of A β , mutant APP itself or APP fragments after β -secretase cleavage have also been suggested to induce neurotoxicity. Several reports in the literature for example, have claimed that the C99 fragment of APP induces neuron loss.^{227,228} To further complicate the issue, another recent study indicated that mutant PS1 might be responsible for neuron loss.²²⁹ It thus remains to be clearly established which molecule(s) is (are) connected with age-related loss of neurons *in vivo*. The major candidates are summarized in Figure 6. An important confounding factor in most of the discussed studies is that quantification of neuronal numbers (to assess reductions in neuronal numbers) was carried out with conventional quantitative methods which are subjected to considerable bias and therefore do not answer the research questions accurately.^{140,230} Only a limited number of studies was carried out with appropriate

methodology in this respect, i.e. modern design-based stereology which allows for precise quantification of the total numbers of neurons and possible changes thereof.^{140,230}

The issues raised above on the identification of the molecules responsible for age-related loss of neurons in AD are also applicable for the identification of the molecules responsible for age-related loss of synapses.^{155,231} The high importance of synaptic alterations for AD pathogenesis is based on the observations that synaptic alterations precede frank signs of neuron loss in AD pathogenesis,¹⁵⁵ and that loss of synapses correlates well with cognitive decline in AD patients.²³² The identification of the molecules responsible for age-related synapse loss in AD thus also awaits elucidation.

The pathogenesis of sporadic AD has been proposed to largely overlap with that of familial AD, although the influence of aging and the ApoE-genotype are much more pronounced in sporadic AD. For the pathogenesis of sporadic AD, where specific mutations in APP and PS cannot account for the pathogenesis, recent evidence may show out to be of high relevance. These studies suggest that age-related oxidative stress mediates alternate APP processing towards A β production,²³³ that aging is associated with higher levels of BACE activity,²³⁴ and that aging is associated with a reduced neprilysin activity.²³⁵ The ApoE4 genotype has furthermore been reported to enhance A β generation and increase oxidative stress although the underlying mechanism remains largely unclear.²³⁶

Despite the debates about the exact molecules responsible for neuronal and synaptic loss and the exact temporal sequence of the A β cascade, evidence in favor of the A β cascade hypothesis is accumulating.^{162,164} As a result, the A β cascade hypothesis is currently considered to be the main hypothesis on AD pathogenesis.^{72,162,164} Nonetheless, the A β cascade hypothesis has remained controversial. One reason for this controversy is the lack of clear evidence of neuron loss in transgenic mice with abundant A β formation.⁷¹ A second reason is the observation that the burden of neuritic plaques does not correlate with the clinical phenotype in AD patients whereas occurrence of NFT and reductions in neuronal or synaptic numbers do.^{73,237} In alternative hypotheses on AD pathogenesis, tau protein abnormalities,^{238,239} heavy metals,^{240,241} vascular factors,²⁴² and disturbed cholesterol homeostasis²⁴³ have been proposed as central players. It should be noted that these hypotheses are alternative but not mutually exclusive. When appropriately used and interpreted with caution, animal models can be very useful in deciphering the contributions of proposed molecules and mechanisms.

In summary, although several genetic, molecular and biological investigations argue for the A β cascade hypothesis, this hypothesis and its temporal sequence of events involved remain to be appropriately tested *in vivo*. Ultimately, the complex pathogenetic changes involved in AD result in neuritic dystrophy, loss of neurons and synapses and an activation of the immune system.

QUESTIONS AND HYPOTHESES ADDRESSED IN THIS THESIS

- i) The development of modern design-based stereology has prompted many neuroscientists (especially those working on alterations of neuron numbers) to use this methodology in investigations of neuronal death/loss. However, two important questions have remained unaddressed in the use of stereology. Does the analysis of sets of sections systematically and randomly sampled spanning the entire brain regions of interest (SRS series) per animal lead to other results than the analysis of one single section per animal? Can groups of animals show different mean total numbers of neurons but similar mean neuronal densities, which would limit the impact of neuronal density data in investigations of neuronal death/loss? To address this question, quantifications of neuron numbers and regional volumes were performed on SRS series and the obtained results were compared to data obtained by parallel analyses on a single "representative" section per animal. I hypothesized that data obtained by analyzing SRS series would substantially differ from data obtained by analyzing a single "representative" section per animal and that therefore conclusions based on these results differ as well. Furthermore, I hypothesized that results analyzed and expressed as total numbers of neurons can substantially differ from results analyzed and expressed as neuronal densities and may thereby lead to different conclusions on the same material. These hypotheses were tested in the study described in Chapter 2.

- ii) The mechanisms which underlie the vulnerability of selective types of neurons in the aging brain remain largely unknown. Previous studies have shown a striking cell-type-specificity of age-related decline in the rate of nDNA repair during aging in the mouse brain. In Section 2.3. of this Chapter and in Chapter 3, I hypothesized that types of neuron with an age-related decline in the rate of nDNA repair show an accumulation of unrepaired nDNA damage during aging without an age-related loss of neurons whereas types of neuron without an age-related decline in the rate of nDNA repair show no accumulation of unrepaired nDNA damage during aging but do show an age-related loss of neurons. This hypothesis was tested for the first time in a controlled study on the same species, i.e., a cohort of aging mice of the same strain was investigated cell-type-specifically for age-related accumulation of unrepaired nDNA damage and age-related reductions in numbers of neurons. In Chapter 4 the study is described to test the hypothesis on four distinct types of neurons in the aging mouse brain, i.e. hippocampal pyramidal cells, hippocampal granule cells, cerebellar granule cells and cerebellar Purkinje cells.

- iii) A major line of evidence has indicated that increased production of free radicals and oxidative stress are central events in aging.² Current evidence on the role of oxidative stress in aging and AD and the potential value of the use of endogenous and exogenous

antioxidants as a preventative strategy for AD are discussed in Chapter 5. Another strategy, caloric restriction, has been known for years to extend mean and maximum life span in a variety of species, although little is known about the exact effects of caloric restriction on the aging process in the brain, and on potential differential effects of caloric restriction on different brain regions and types of neurons in the brain during aging? On the basis of these issues, I hypothesized that a combination of exogenous and endogenous antioxidants with caloric restriction is a good strategy to attenuate age-related alterations in the brain. Specifically, I hypothesized that upregulation of an endogenous antioxidant (by transgenic overexpression of human Cu/Zn-superoxide dismutase in mice) together with a diet restricted in calories and enriched in exogenous antioxidants would attenuate age-related alterations in the hippocampus. Chapter 6 describes the study to test this hypothesis on age-related morphologic and molecular alterations in the mouse hippocampus.

- iv) Despite general agreement that the A β cascade hypothesis is the main one for AD pathogenesis, it remains unclear whether any of the molecules – and if so which – mentioned in the cascade is responsible for age-related loss of neurons and synapses *in vivo*. I hypothesized that intraneuronal A β rather than the other candidates (Figure 6) is connected with age-related loss of hippocampal neurons and synaptophysin-immunoreactive-presynaptic boutons. With the aim to assess age-related effects of the mentioned candidates (Figure 6), a systematic investigation was carried out to find out which molecules of the A β pathway are involved in synapse and neuron loss. Chapter 7 focuses on the question whether neuronal expression of C99 fragment results in age-related hippocampal neuron and synapse loss, Chapters 8 on the question whether extracellular A β aggregations in the form of plaques, intraneuronal A β or mutant PS1 result in age-related hippocampal neuron loss. Next, Chapter 9 focuses on the question whether mutant APP, mutant PS1, extracellular A β aggregations or other A β -dependent events are associated with age-related synapse loss. I hypothesized that the C99 fragment of APP, mutant APP itself and mutant PS1 itself do not result into loss of hippocampal neurons or synapses, whereas both extracellular A β aggregations and intracellular A β do play important roles in hippocampal neuron and synapse loss. The corresponding investigations were carried out with a set of novel transgenic mice.
- v) As mentioned in section 3.6., neuropathologic alterations in AD affect selective regions and types of neurons such as pyramidal neurons in the neocortex and hippocampus. These same regions and types of neurons are known to be affected in normal aging, although to a lesser degree. From this similarity the question arises whether the degenerative processes occurring during normal aging are the same as those occurring in AD? In this respect, I hypothesized that degenerative processes that occur during aging set the stage for degenerative events involved in AD pathogenesis, but that the actual cellular mechanism which underlie AD are different than those which underlie aging. More specifically, I hypothesized that aging is associated with increased oxidative stress, accumulation of DNA damage, and moderate synaptic disturbances, that these age-related events are the ‘sine-qua-non’ for AD pathogenesis but that the neurode-

generative events in AD far surpass and are partly different from those in aging. In particular, I hypothesize that alternate processing of APP, intraneuronal accumulation of A β within the endolysosomal system with subsequent disturbances in cellular function and viability are events that are not substantially involved in normal aging but do play crucial roles in AD. These hypotheses were tested in different experiments which are described in several chapters throughout this thesis. The results from the experiments testing these hypotheses are discussed in Chapter 10.

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Chapter 2

Prenatal protracted irradiation at very low dose rate induces severe neuronal loss in rat hippocampus and cerebellum

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ABSTRACT

Prenatal irradiation is known to damage the developing brain. However, little is known about the consequences of very low dose rate prenatal protracted irradiation over several days on neuron numbers in the offspring brain, and on volumes of the corresponding brain regions. Pregnant Wistar rats were exposed either to a protracted gamma irradiation from embryonic day (E) 13 to E16 (0.7 mGy/min; total cumulative dose approximately 3 Gy) or were sham-irradiated. Thirty months old male and female offspring were then analyzed for alterations in hippocampal and cerebellar morphology. Using design-based stereology and the analysis of sets of sections systematically and randomly sampled to span the entire brain region of interest, a statistically significant decrease in numbers of hippocampal pyramidal and granule cells as well as of cerebellar Purkinje and granule cells (approximately 50%) was found in male and female irradiated offspring. The volumes of these brain regions were comparably altered. The analysis of only a "representative" section per animal yielded mostly non-significant trends. Evaluation of neuron densities showed no differences between prenatally irradiated and sham-irradiated offspring. Most importantly, very low dose rate prenatal protracted gamma irradiation did not result in the same morphologic alterations in the offspring brain as previously observed after prenatal single irradiation such as derangement of the laminar structure of pyramidal cells within the hippocampus or malformation of cerebellar lobules.

INTRODUCTION

Lesions of the central nervous system are a common finding in offspring following prenatal exposure to ionizing radiation.¹⁻³ Severity of damage and vulnerability to radiation are a function of dose, dose rate and gestational age of exposure.⁴⁻⁶ For example, single X-irradiation with high dose rates (i.e., application of doses higher than 1 Gy within a few minutes) leads to dramatic effects on brain integrity.⁷⁻⁹ Single prenatal low-dose X-irradiation (i.e., single X-irradiation with application of doses lower than 1 Gy within a few minutes) results in more discrete alterations in postnatal brain morphology such as clusters of pyknotic neurons scattered amongst normal hippocampal neurons,⁹ delayed loss of hippocampal pyramidal cells during adulthood¹⁰ or discrete alterations in the spatial arrangement of cortical pyramidal cells.¹¹ Studies employing fractionated irradiation (i.e., two or more irradiation sessions during pregnancy, with application of doses higher or lower than 1 Gy within a few minutes each) were either focused on exposure during early gestational periods,¹² on structural brain anomalies expressed prenatally or early postnatally,¹³⁻¹⁵ or on the comparison between a single high-dose irradiation and two doses a few hours apart with the same total cumulative dose.¹⁶ In contrast, the consequences of prenatal protracted application of ionizing radiation at constant, very low dose rate over several days on the developing brain remain poorly known. Few alterations have been documented, such as slightly reduced brain weight or a reduction in the volume of the cingulum bundle.¹⁷ This limited knowledge about effects of very low dose rate prenatal protracted irradiation (VLDRPP irradiation) on the brain, however, stands in contrast to the potential social relevance of this kind of radiation particularly in relation to issues as varied as the future of nuclear power, manned space exploration, and radiological terrorism.¹⁸

The aim of this study was to assess for the first time long-term effects of VLDRPP irradiation on neuron numbers and regional volumes within the offspring's brain. Pregnant rats were irradiated from embryonic day (E) 13 to E16, which is in the period during which both the hippocampal pyramidal cells and cerebellar Purkinje cells begin to differentiate in the rat brain.^{6,19,20} Both cell types play an important role in normal brain development and maturation, and at least the cerebellar Purkinje cells may have an inductive effect on the development of the cerebellar granule cells which are generated postnatally.²¹⁻²³ VLDRPP irradiation was carried out at a dose rate of 0.7 mGy/min (total cumulative dose 3 Gy). A comparable dose rate and total cumulative dose (1 mG/min for 25 h; total cumulative dose 1.5 Gy) was recently applied to human cells *in vitro* in a study assessing the risk of space crews by space irradiation,²⁴ to give an example.

In addition, this study addressed a methodological question, i.e., to which extent analyses of neuron numbers and regional volumes within the brain depend on the selection of sections to be analyzed. To do so the quantifications were performed using design-based stereology, and sets of sections systematically and randomly sampled spanning the entire brain regions of interest (SRS series) were analyzed (for review see ref. 25). The obtained results were compared to data obtained by parallel analyses on a single "representative" section per animal. Furthermore, alterations in neuron numbers were compared to alterations in neuron densities.

MATERIALS AND METHODS

Animals and VLDRPP irradiation

Ten timed pregnant Wistar rats were kept at the Department of Radiobiology of the Belgian Nuclear Research Centre (SCK-CEN, Mol, Belgium; air-conditioned rooms, 20° C, 60% relative humidity, 12:12 hours light:dark cycle with artificial lights on at 6:00 a.m., ad libitum access to water and standard laboratory chow). From E13 to E16 (E0: mating between 06.00 and 08.30 p.m.), five animals were subjected in a bunker to VLDR protracted irradiation under the following conditions: cobalt-60 gamma source without filters; 72 hours protracted irradiation; average expected dose rate 0.7 mGy/min (average expected total cumulative dose 3 Gy); cages placed in a panoramic configuration (i.e., on a circle) with the source at the center position; distance between cages and source 139 cm; air-conditioned room, 21±1° C, 60% relative humidity; lights off during the whole exposure except of a red light from an alarm system; ad libitum access to water and standard laboratory chow; animals were free to move in their cages. Each cage was equipped with two lithium fluoride TLD 700 dosimeters (Thermo Electron, Beenham, UK), one fixed on the front wall (i.e., the cage wall facing the source) and one on the back wall. Recorded values were 4.66±0.14 Gy (front wall; mean±S.E.M.) and 2.65±0.05 Gy (back wall). The remaining five animals were kept in the neighbouring room under identical conditions but were not irradiated (sham-irradiation). Birth of pups took place at E22. Immediately after birth, the number of pups per mother was reduced to 10, and weaning took place at postnatal day 25. For the present study, a total of 20 offsprings (one male and one female offspring per dam) were kept at SCK-CEN, and were observed for the development of tumors until they reached the

age of 30 months. All investigations were carried out in strict accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Histology

At 30 months of age, the 20 offsprings were anesthetized at SCK-CEN with chloral hydrate (10% aqueous solution, 0.005 ml/g body weight, intraperitoneal injection), and were sacrificed 5 minutes later by intracardial perfusion–fixation (250 ml phosphate buffered saline solution [PBS; pH 7.4, 37° C], followed by 250 ml formalin solution [10%, 4° C]). The brains were removed from the skulls and postfixed for 10 days in 10% formalin solution at 4° C. Then, they were sent to the Department of Anatomy and Cell Biology, RWTH Aachen University (Aachen, Germany) where they were hemisected exactly in the mediosagittal line. The left cerebellum and brainstem were separated from the rest of the brain by a cut in the coronal plane. Both tissue blocks (left cerebellar half, rest of the left brain half) were cryoprotected in sucrose solution (10%, 20% and finally 30% sucrose in 0.1 M Tris–HCl buffer, 2 × 12 hours per solution; 4° C), and were frozen in Tissue-tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands). Blocks were stored at -70° C until further processing. Blocks containing the left cerebellar half were exhaustively cut into serial, 100 µm–thick sagittal sections on a cryostat (type HM 500 OMV, Microm, Walldorf, Germany) using 80 mm disposable steel blades (type C35, Feather, Japan). Blocks containing the rest of the left brain half were exhaustively cut into serial, 100 µm–thick horizontal sections. Every third section was selected with a random start, mounted on gelatinized glass slides, dried, defatted with Triton X-100 (0.025%, 20 minutes; Merck, Darmstadt, Germany), and stained with cresyl violet (0.01%, 20 minutes). Slides were coverslipped using DePeX (Serva, Heidelberg, Germany).

Stereologic Analysis

For the hippocampus the following parameters were analyzed on a SRS series of sections spanning the entire left hippocampus: volumes of the granule cell layer and the pyramidal cell layer (CA1–3), total numbers and densities of granule cells and pyramidal cells (CA1–3), and the ratio between the number of granule cells and the number of pyramidal cells. In one parallel analysis the following parameters were analyzed on one “representative” section per animal each, with the section showing the largest total projection area of the hippocampal cell layers (dentate gyrus plus CA1–3 fields) as “representative section” (referred to as hippocampal largest projection area section thereafter): projection areas of the granule cell layer and the pyramidal cell layer (CA1–3), and numbers and densities of granule cells and pyramidal cells (CA1–3). In another parallel analysis these parameters were analyzed on the section through the mid body of the left hippocampus as “representative section” of each animal (referred to as hippocampal midbody section thereafter).

Analysis of the cerebellum on a SRS series of sections spanning the entire left cerebellar half comprised the following parameters: volumes of the white matter, granule cell layer and molecular layer, total number of Purkinje cells, total number and density of granule cells, and

Figure 1. Low-power photomicrographs of 100 μm -thick horizontal sections from the left brain half of male (A,B) and female (C,D) rats subjected either to protracted gamma irradiation (0.7 mGy/min; 3 Gy in total) from embryonic day (E) 13 to E 16 (B,D), or to sham-irradiation (A,C). Sections were stained with cresyl violet and show the mid-body of the left hippocampus (scale bar = 1.5 mm). Note the smaller size of the hippocampus and the cerebral cortex, as well as the larger ventricles, in the prenatally irradiated animals compared to the sham-irradiated ones. The insets show high-power photomicrographs of the pyramidal cell layers (CA3 field) and the granule cell layers (dentate gyrus; DG), and are representative of the magnification at which the stereologic estimates were obtained (scale bar = 15 μm). The small squares indicate the positions at which the insets were taken. Note the similar size, shape, distribution and density of the neurons among the prenatally irradiated and the sham-irradiated animals

Figure 2. Low-power photomicrographs of 100 μm -thick parasagittal sections from the cerebellum of male (A,B) and female (C,D) rats subjected either to protracted gamma irradiation (0.7 mGy/min; 3 Gy in total) from embryonic day (E) 13 to E 16 (B,D), or to sham-irradiation (A,C). Sections were stained with cresyl violet and show the same parasagittal level in cerebellum and brainstem (scale bar = 1.5 mm). Note the smaller size of the cerebellum of the prenatally irradiated animal compared to the sham-irradiated one, as well as the smaller size of the cerebellum of the sham-irradiated female rat compared to the sham-irradiated male. Interestingly, prenatal irradiation affected the branching pattern, but not the numbers, of the cerebellar lobules. The insets show high-power photomicrographs of the border zone between the molecular layer (on the left), the Purkinje cell layer (middle) and the granule cell layer (on the right), and are representative of the magnification at which the stereologic estimates were obtained (scale bar = 15 μm). Note the similar size, shape, distribution and density of the neurons among the prenatally irradiated and the sham-irradiated animals.

Figure 3. Results of the stereologic investigations in the hippocampus. Analysis of male (squares; 'M' in Q, R, S) and female (circles; 'F' in Q, R, S) rats subjected to prenatal gamma-irradiation (open squares and circles; 'X' in Q, R, S) or to sham-irradiation (black squares and circles; 'S' in Q, R, S). Data were obtained by analyzing a systematic-random series of horizontal sections spanning the entire left hippocampus (column on the left), by analyzing a horizontal section of the left hippocampus showing the largest total area of hippocampal cell layers (dentate gyrus [DG] plus CA1-3) in each animal (column in the middle), or by analyzing a horizontal section through the mid body of the left hippocampus in five animals per group. Data are shown as individual values and means per group (lines), and are given as volumes in A and D, projection areas in B, C, E and F, total numbers of neurons in G and N, numbers of neurons per section in H, I, O and P, and numbers of neurons per unit volume in K, L, M, Q, R and S. ANOVAs revealed significant differences between the groups in A, B, D, G, H, N, O ($p < 0.001$), I ($p = 0.003$) and C ($p = 0.011$). The p values from the corresponding post-hoc Bonferroni's multiple comparison tests are provided as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) in A, B, C, D, G, H, I, N and O. ANOVAs did not show significant differences between the groups in E, F, K, L, M, P, Q, R and S ($p > 0.05$).

the ratio between the number of granule cells and the number of Purkinje cells. As for the hippocampus parallel analyses were performed on one "representative" section per animal each, with either the section showing the largest total cerebellar projection area (referred to as cerebellar largest total projection area section thereafter) or the first paramedian sagittal section of the left cerebellar half (referred to as cerebellar first paramedian section thereafter) as "representative section". In these parallel analyses the following parameters were investigated: projection areas of the white matter, granule cell layer and molecular layer, numbers of Purkinje and granule cells, and density of granule cells.

The hippocampal midbody section and the cerebellar first paramedian section in each animal were selected by microscopic pilot inspection of all sections of the corresponding SRS series of sections. The hippocampal largest projection area section and the cerebellar largest total projection area section in each animal were selected after evaluating these parameters for all sections of the corresponding SRS series of sections. Stereologic analyses were performed at the Department of Anatomy and Cell Biology, RWTH Aachen University (Aachen, Germany) with a stereology workstation, consisting of a modified light microscope (BX50; Olympus, Tokyo, Japan), Olympus UPlanApo objectives (20 $\times$, NA = 0.70; 40 $\times$, oil, NA = 1.00; 100 $\times$, oil, NA = 1.35), motorized specimen stage for automatic sampling (Märzhäuser, Wetzlar, Germany), electronic microcator (Heidenhain, Traunreut, Germany), CCD colour video camera (JAI, Glostrup, Denmark), PCs with frame grabber boards (Screen Machine II, Fast Multimedia, Munich, Germany; or Flashpoint Intrigue light, Integral Technologies, Indianapolis, IN), stereology software (C.A.S.T.-Grid, Olympus; StereoInvestigator, MicroBrightField, Williston, VT), and a 17" television screen monitor. Projection areas of hippocampal cell layers were investigated by tracing the boundaries of these layers on video images displayed on the computer, whereas projection areas of cerebellar layers were analyzed by point counting methods. From the obtained data and the corresponding average actual section thickness after histologic processing (measured with the electronic microcator), the volumes of hippocampal granule cell layer and pyramidal cell layer (CA1–3) and of cerebellar white matter, granule cell layer and molecular layer were calculated using Cavalieri's principle.²⁶ The variability of the estimated volumes of the cerebellar layers was assessed with the method described by Garcia-Finana et al.²⁷ for estimates of volumes based on traced boundaries a procedure to predict precisely the CE is currently not available (see ²⁵ for details).

Neuron numbers were estimated with the optical fractionator.^{25,28–30} All neurons whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the brain regions of interest were counted.²⁵ Estimated numbers of neurons per section and estimated total numbers of neurons per brain half were calculated from the numbers of counted neurons per section (or per brain half, respectively) and the corresponding sampling probabilities. For estimates of the number of neurons per section the section sampling fraction (ssf) was 1, whereas ssf was 3 when calculating estimated total numbers of neurons per brain half as every third section was selected for analysis.^{25,28–30}

Estimates of section-specific neuronal densities were calculated by dividing the number of neurons in a given cell layer by the product of the projection area of this cell layer and the

actual section thickness of the corresponding section after histologic processing. Estimates of global neuronal densities were calculated by dividing the total number of neurons of a given cell type by the total volume of the corresponding cell layer. As it was not possible to delineate exactly the boundaries of the cerebellar Purkinje cell layer in the rat brain, estimates of Purkinje cell densities were not calculated. Finally, for each investigated brain region the individual frequency distributions of the number of neurons per unbiased virtual counting frames were calculated, as well as the corresponding mean group data. Details on the counting procedures are summarized in Appendix. To control for litter effects, the ratios of the individual results for male and female offsprings of a given dam were calculated (only in the case of results obtained from a SRS series of sections).

Statistical analysis

For each group of animals, mean and standard error of the mean were calculated for all investigated parameters. Comparisons between groups were performed with one-way analysis of variance (ANOVA), followed by post-hoc Bonferroni's multiple comparison tests for pairwise comparisons. Differences in mean ratios of the individual results for male and female offsprings of a given dam between prenatally irradiated and prenatally sham-irradiated animals were tested with Student's unpaired *t* test. Statistical significance was established at $p < 0.05$. All calculations were performed using GraphPad Prism (Version 4.00 for Windows, GraphPad Software, San Diego, CA).

Photography

All photomicrographs were produced by digital photography using an Olympus U-CMAD-2 digital camera attached to an Olympus AX 70 microscope and image analysis software (analySIS; Imaging System, Münster, Germany). The final figures were constructed using Corel Photo-Paint v. 11 and Corel Draw v. 11. Only minor adjustments of contrast and brightness were made, which in no case altered the appearance of the original materials.

RESULTS

Histology

The brains of prenatally irradiated animals were smaller than the brains of prenatally sham-irradiated ones with smaller size of cortex, hippocampus, midbrain and cerebellum. Furthermore, the prenatally irradiated animals had considerably larger lateral ventricles than the prenatally sham-irradiated ones. In spite of the considerable decrease in size, the neocortex, hippocampus and cerebellum of the prenatally irradiated animals did not show obvious alterations in cytoarchitecture compared to the prenatally sham-irradiated animals. In addition, the prenatally irradiated animals showed alterations in the branching pattern, but not in the number, of the cerebellar lobules (Figs. 1 and 2). None of the investigated animals developed brain tumors (identified by histology in the course of the investigations) or other tumors evident at routine inspection following perfusion-fixation, respectively.

Figure 4. Ratios of total neuron numbers in hippocampus and cerebellum.

Data are shown as individual values and means per group (lines). HG, hippocampal granule cells; HP, hippocampal pyramidal cells; CG, cerebellar granule cells; CP, cerebellar Purkinje cells. M-S, male rats subjected to prenatal sham-irradiation; M-X, male rats subjected to prenatal irradiation; F-S, female rats subjected to prenatal sham-irradiation; F-X, female rats subjected to prenatal irradiation.

Stereologic analysis of the left hippocampus

When analyzing an SRS series of sections spanning the entire left hippocampus per animal, the following results were obtained: rats subjected to prenatal irradiation showed a statistically significant decrease in the mean volumes of both granule and pyramidal cell layers compared to the rats subjected to prenatal sham-irradiation (Figure 3A, D), as well as a significant decrease in the mean total numbers of both granule and pyramidal cells (Figure 3G, N), independent of sex. In addition, the prenatally sham-irradiated female rats had on average significantly fewer granule cells than the prenatally sham-irradiated males (Figure 3G). In contrast to total numbers, the densities of both granule and pyramidal cells were unaffected by the prenatal irradiation (Figure 3K, Q). The ratio between the number of granule cells and the number of pyramidal cells was 1.34 on average and showed no significant difference between the groups (Figure 4). The ratios of individual results for male and female offsprings of a given dam showed considerable differences among dams. However, there were no differences in mean values of these ratios between irradiated and sham-irradiated dams (Figure S1 in Appendix). Compared with these SRS series of sections data, the corresponding hippocampal largest projection area sections (Figure 3B, E, H, L, O and R) indicated that only male rats subjected to prenatal irradiation had a significant decrease in the mean projection area of the granule cell layer and the mean number of granule cells compared to rats subjected to prenatal sham-irradiation (Figure 3B, H). Furthermore, prenatally sham-irradiated female rats had a significantly smaller mean projection area of the granule cell layer than prenatally sham-irradiated males (Figure 3B). Also, the irradiation-induced reduction in the mean projection area of the pyramidal cell layer did not reach statistical significance (Figure 3E). The hippocampal midbody section results showed major differences with the corresponding SRS series of sections results. Specifically, only male but not female rats subjected to prenatal irradiation showed a statistically significant decrease in the mean projection area of the granule cell layer and in the mean number of granule cells (Figure 3C, I). Furthermore, prenatal irradiation had no significant effect on both the mean pro-

Figure 5. Results of the stereologic investigations in the cerebellum. Analysis of male (squares; 'M' in Q,R,S) and female (circles; 'F' in Q,R,S) rats subjected to prenatal gamma-irradiation (open squares and circles; 'X' in Q,R,S) or to sham-irradiation (black squares and circles; 'S' in Q,R,S). Data were obtained by analyzing a systematic-random series of sagittal sections throughout the left cerebellar half (column on the left), analyzing the first paramedian sagittal section of the left cerebellar half (column in the middle), or evaluating a sagittal section of the left cerebellar half showing the largest total cerebellar projection area (column on the right) in five animals per group. Data are shown as individual values and means per group (lines), and are given as volumes in A, D and G, projection areas in B, C, E, F, H and I, total numbers of neurons in K and N, numbers of neurons per section in L, M, O and P, and numbers of neurons

*per unit volume in Q, R and S. ANOVAs revealed significant differences between the groups in A, B, D, E, G, H, K, L, and N ($p < 0.001$), C ($p = 0.006$), F ($p = 0.017$), M ($p = 0.018$) and I ($p = 0.046$). The p values from the corresponding post-hoc Bonferroni's multiple comparison tests are provided as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) in A to H, K, L and N. ANOVAs did not show significant differences between the groups in O to S ($p > 0.05$).*

jection area of the pyramidal cell layer and the mean number of pyramidal cells (Figure 3F, P). Interestingly, female rats subjected to prenatal irradiation showed a substantial increase in the density of pyramidal cells compared to the female rats subjected to prenatal sham-irradiation. This effect however, was not statistically significant due to very high interindividual variation (Figure 3S).

Stereologic analysis of the left cerebellar half

In general, trends in irradiation-induced morphologic alterations comparable to those found in the hippocampus were obtained in the cerebellum when analyzing an SRS series of sections spanning the entire left cerebellar half per animal. Specifically, both male and female rats subjected to prenatal irradiation showed a significant decrease in the mean volumes of the white matter, granule cell layer and molecular layer compared to rats subjected to prenatal sham-irradiation (Figure 5A, D, G) as well as a significant decrease in the mean total numbers of both Purkinje and granule cells (Figure 5K, N). In addition, prenatally sham-irradiated female rats had significantly smaller mean volumes of the white matter, granule cell layer and molecular layer than prenatally sham-irradiated males (Figure 5A, D, G). The density of the granule cells was not affected by prenatal irradiation (Figure 5Q). The ratio between the number of granule cells and the number of Purkinje cells was 333 on average and showed no significant difference between the groups (Figure 4). As for the hippocampus, the ratios of individual results for male and female offsprings of a given dam showed considerable differences among the dams but no significant differences in mean values (Figure S1 in Appendix).

Compared with these SRS series of sections data, there was no significant difference in the mean number of granule cells between prenatally irradiated and prenatally sham-irradiated animals on the largest total projection area sections (Figure 5O), as well as no significant difference in the mean projection areas of both granule cell layer and molecular layer between the prenatally sham-irradiated male and female rats (Figure 5E, H).

When the analysis was restricted to the first paramedian sagittal section of the left cerebellar half the results showed major differences with the corresponding data obtained by analysis of SRS sections spanning the entire left cerebellar half. Specifically, significant differences between prenatally irradiated and prenatally sham-irradiated rats were only found for male rats in the mean projection area of the cerebellar white matter (Figure 5C) and for female rats in the mean projection area of the cerebellar granule cell layer (Figure 5F). Differences in mean numbers of cerebellar Purkinje and granule cells between prenatally irradiated and prenatally sham-irradiated animals did not reach statistical significance (Figure 5M, P).

DISCUSSION

This is the first detailed investigation of the effects of VLD RPP irradiation on neuron number in the brain of offsprings using design-based stereologic methods. VLD RPP gamma irradiation of rats with a dose rate of 0.7 mGy/min from E13 to E16 (total cumulative dose 3 Gy) resulted in a massive decrease in the total numbers of hippocampal and cerebellar neurons (approximately 50% on average) in male and female irradiated offspring, with comparable alterations in the volumes of these brain regions. The mean total numbers of hippocampal and cerebellar neurons of the prenatally sham-irradiated (i.e., control) animals (Figure 3 and 5), the mean ratio between the number of hippocampal granule cells and the number of hippocampal pyramidal cells (Figure 4) and the mean ratio between the number of cerebellar granule cells and the number of cerebellar Purkinje cells (Figure 4) of both prenatally irradiated and sham-irradiated animals were in line with several reports on neuron numbers in rats.^{31–38} This demonstrates the validity of the obtained results. Considerable differences among dams in ratios of individual results for male and female offsprings of a given dam (Figure S1) indicate that the observed effects were attributable to prenatal irradiation and did not represent litter effects (in case of litter effects the offsprings of a given dam would have shown similar effects, and the ratios of individual results for male and female offsprings of a given dam would have been similar among dams).

Methodological considerations

This study demonstrates remarkable differences between data obtained by analyzing sets of sections systematically and randomly sampled spanning the entire brain regions of interest (SRS series) and data obtained by analyzing a single “representative” section per animal. For both hippocampus and cerebellum the latter procedure resulted in less pronounced differences between the investigated groups of animals. In consequence, for many investigated parameters merely (non-significant) trends were observed when analyzing a “representative” section per animal, while the corresponding analysis of SRS series showed statistically significant differences between the groups. This was connected with a trend towards increased interindividual variability in the data obtained by analyzing a “representative” section per animal. The reason for this was most probably an increased intra-individual variability, that is, the variation of the individual data due to the limited number of analyzed sections (as shown in^{29,39,40}). Interestingly, the largest projection area section results were in better concordance of the SRS series of sections results than the midbody section results (in case of the hippocampus) and the first paramedian section results (in case of the cerebellum, respectively). In one case (investigation of the size of the hippocampal granule cell layer) the midbody sections even showed a statistically significant difference between the prenatally sham-irradiated male and female rats, which was not found when SRS series of sections were investigated (Figure 3A and 3B).

Finally, there was a striking difference between massive alterations in neuron numbers and an almost lack of alterations in neuron densities as a consequence of prenatal irradiation. This difference is due to the fact that prenatal irradiation resulted not only in neuron loss but also in a massive decrease in volume of the investigated brain regions. Note that the reverse situation has been described in the literature, with statistically significant reductions in neuron densities

without concomitant alterations in neuron numbers due to increased volumes of the investigated brain regions.^{41–44} Thus, the data presented here show how results obtained using design-based stereology can be influenced by the selection of sections containing the brain region of interest, and how the outcome of studies can differ when focussing on neuron densities instead of neuron numbers. As already mentioned by West,⁴⁵ the uncritical use and interpretation of neuron density data should be particularly disapproved. Furthermore, it might be useful to base estimates of the volume of brain regions and of neuron numbers on the analysis of sets of sections systematically and randomly sampled spanning the entire brain regions of interest, whenever possible.

Differences between the effects of VLDRPP irradiation and single prenatal irradiation on the offsprings' brain

Importantly, the VLDRPP irradiation protocol applied in the present study did not result in the same morphologic alterations in the offsprings' brain as previously observed after prenatal single irradiation with high dose rates. For example, we did not observe a derangement of the laminar structure of pyramidal cells within the hippocampus, as reported after single X-irradiation of rats with a dose of 1.5 Gy on E15.⁴⁶

Neither we found a four-layered 'lissencephalic' cortex as seen after single X-irradiation of rats with a dose of 2 Gy on E16⁴⁷ nor abnormal hippocampal and cerebellar cortical cellular patterns after single X-irradiation of rats with a dose of 2 Gy on E17,⁴⁸ or the formation of anteroposteriorly and horizontally directed cerebellar lobules, as opposed to the normal transverse arrangement, after single X-irradiation of rats with a dose of 2 Gy on E16.⁴⁸ Thus, the effects of VLDRPP irradiation on the developing brain differed from the effects of prenatal single irradiation with high dose rates.

Effects of VLDRPP irradiation on the offsprings' brain

To date few studies have focussed on the effects of VLDRPP irradiation on the offspring brain. Reyners et al.¹⁷ reported a statistically significant reduction in adult brain weight by 13% after VLDRPP gamma irradiation of rats with a dose rate of 0.1 mGy/min from E12 to E16 (total cumulative dose 0.56 Gy), as well as by 7.3% after VLDRPP gamma irradiation with a dose rate of 0.1 mGy/min from E14 to E20 (total cumulative dose 0.8 Gy). For the latter group of animals, Reyners et al.¹⁷ found also a significant 19% reduction in the volume of the cingulum bundle, potentially indicating neuron loss in the brain of the offsprings. Wang et al.⁴⁹ subjected pregnant mice from E13 to E18 (corresponding to about E14.5 to E20.5 in rats⁵⁰) to VLDRPP gamma irradiation with dose rates of 0.02, 0.03 or 0.056 mGy/min, respectively (total cumulative dose 0.1, 0.2 or 0.4 Gy, respectively) and found slight irradiation-induced alterations in various behavioral tasks in the adult offspring. However, the authors did not analyze the brains of these animals. In a study focussing on the migration of neuronal cells in the developing cerebral cortex, Hyodo-taguchi et al.⁵¹ injected pregnant mice with bromodeoxyuridine (BrdU) on E14 and exposed them from E14 to E17 (corresponding to about E15.5 to E19 in rats⁵⁰) to VLDRPP gamma irradiation with dose rates of 0.08, 0.23 or 0.71 mGy/min, respectively (total cumulative dose 0.3, 0.9 or 2.8 Gy, respectively). VLDRPP irradiation with dose rates of 0.08

mGy/min and 0.23 mGy/min had no significant effect on the migration of cortical neurons, whereas VLDRPP irradiation with 0.71 mGy/min resulted in delayed migration of cortical neurons during the embryonic period and significant differences in the distribution pattern of BrdU-labeled cells within the cerebral cortex relative to control animals at eight weeks of age. Furthermore, Hyodo-taguchi et al.⁵¹ observed in three-week-old animals subjected to VLDRPP irradiation with a dose rate of 0.71 mGy/min a significantly decreased number of BrdU-labeled cells within the cerebral cortex (approximately -40% on average compared to control animals). By contrast, in one-day old animals subjected to the same irradiation protocol the number of BrdU-labeled cells within the cerebral cortex showed no significant difference between prenatally irradiated and prenatally sham-irradiated animals. Hyodo-taguchi et al.⁵¹ interpreted this finding as suggestive of irradiation-induced neuron death during the postnatal maturation of the cerebral cortex. However, this interpretation should be considered with some caution since the analysis was performed on merely a few sections per animal (coronal brain sections covering the rostral portion of the hippocampus) without use of design-based stereologic methods. Furthermore, it cannot be excluded that the reduction in the number of BrdU-labeled cells in the study of Hyodo-taguchi et al.⁵¹ was at least in part caused by the fact that incorporation of BrdU into the DNA of mammalian cells may cause them to become more sensitive to the deleterious effects of ionizing radiation.⁵²⁻⁵⁴

Thus, the present data agree with previous reports using similar irradiation protocols but substantially expand our knowledge of the effects of VLDRPP irradiation on the offsprings' brain. It should be mentioned that in some studies injection of tritiated water into pregnant dams was used to induce protracted prenatal irradiation at low dose rates to the fetuses.⁵⁵⁻⁵⁷ However, it is not possible to compare the results of these studies directly to the present data, since the fetuses are not irradiated at a constant dose rate after injection of tritiated water into pregnant dams. Rather, the dose rate in these conditions shows a fast increase within a few minutes, followed by an exponential decrease over several days.⁵⁷

Mechanisms of damage to the offsprings' brain by VLDRPP irradiation

The VLDRPP irradiation protocol used in the present study resulted in a severe decrease in the numbers of hippocampal pyramidal cells and cerebellar Purkinje cells, whose final progenitors underwent division during the selected period of irradiation.^{6,19,20} Although not demonstrated by the present study, this suggests that the vulnerable period for both types of cells even to our VLDRPP irradiation protocol closely overlaps with the phase of division of their final progenitors, as is known to occur for prenatal single irradiation with high dose rates and short irradiation times.^{6,58,59} In this regard, data from Benekou et al.⁶⁰ and Bolaris et al.⁶¹ are particularly relevant. These authors exposed rats to prenatal single X-irradiation with doses of 0.1, 0.2 or 0.4 Gy on E15 or E17. Even after only 0.1 Gy the authors detected increased numbers of apoptotic as well as p53-immunoreactive cells, downregulation of IGF-I, and a compensatory increase in the expression of IGF-II, BDNF, and NT-3 among proliferating, migratory and postmitotic neurons in the developing neuroepithelium. This indicates the occurrence of an X-irradiation-induced cell death by apoptosis with possible compensatory mechanisms in the developing rat brain (similar results were reported by Wang et al.⁴⁹). Thus, it is possible that the VLDRPP irradiation

protocol used here also caused apoptotic cell death of hippocampal pyramidal cells and cerebellar Purkinje cells in the developing brain. Whether this is related to alterations in the expression levels of p53 and neurotrophic factors remains to be determined. Strikingly, we also observed a substantial decrease in the numbers of hippocampal and cerebellar granule cells, which are mostly generated postnatally.^{62,63} Thus, the vast majority of the final progenitors of these cells did not divide during the selected period of irradiation. One explanation may be that in this case, the progenitors of these cells were damaged by irradiation. This is supported by findings on the spontaneous neurological mutant mouse *dreher*. This mutant mouse shows during embryonic development a disruption of the rhombic lip, which is the source of cerebellar granule cell progenitors,^{64,65} as well as in postnatal animals a substantial decrease in the number of cerebellar granule cells.⁶⁶ Another possibility may be irradiation-induced reductions in the amount of growth factors such as Bmp6, Bmp7 and Gdf7, i.e., peptide growth factors of the bone morphogenetic protein (BMP) family. Dorsal midline cells adjacent to the rhombic lip express Bmp6, Bmp7 and Gdf7 which are suggested to initiate the program of cerebellar granule cell specification⁶⁴ and have strong neurotrophic effects on cerebellar granule cells.⁶⁷

On the other hand we did not find irradiation-induced alterations in the ratios between the number of hippocampal granule cells and the number of hippocampal pyramidal cells as well as between the number of cerebellar granule cells and the number of cerebellar Purkinje cells (Figure 4). Although this does not rule out the possibility that the early granule progenitor cell population was reduced by irradiation or the amount of growth factors was diminished, these unchanged ratios suggest that the decrease in the numbers of hippocampal and cerebellar granule cells is due to the absence of trophic support from their main postsynaptic targets, the hippocampal pyramidal cells and cerebellar Purkinje cells. At least for the cerebellum the regulation of the number of granule cells by a predetermined number of Purkinje cells in development has been established in a variety of experimental settings including mouse mutants, toxicologic damage, and prenatal irradiation.^{22,68–71} On the molecular level, sonic hedgehog (Shh) which is produced by Purkinje cells and is required for the proliferation of granule cell precursors plays an important role in this regulation.^{23,72,73} For hippocampal granule cells, the role of trophic support from postsynaptic targets in determining their number is less clear. On the one hand it is known that trophic support from postsynaptic targets is involved in determining neuron numbers in the limbic system as well.⁷⁴ In this study, neonatal rats were subjected to X-irradiation which reduced the number of hippocampal granule cells to 20% of control values.

The number of neurons in both the medial and lateral entorhinal cortices in X-irradiated animals appeared normal until postnatal day [P] 12, at which time a selective reduction in cell numbers became apparent. By P30, approximately 40% of the cells of origin of the perforant path were absent in X-irradiated animals.⁷⁴ The authors concluded that these cells were subject to retrograde transynaptic degeneration as a result of target removal. On the other hand, there is evidence for separate genetic determination of neuron numbers among hippocampal regions.⁷⁵ Furthermore, prenatal ethanol exposure in rats has been shown to reduce the number of hippocampal pyramidal cells without affecting the number of hippocampal granule cells, clearly speaking against a major role of trophic support from postsynaptic targets in determining hippocampal granule cell number.⁷⁶ On the molecular level, basic fibroblast growth factor

(bFGF) has been shown to play an important role in determining the number of granule cells in the developing hippocampus.⁷⁷ Whether there is also a role of Shh in this process remains to be determined.⁷⁸ Interestingly, Shh is involved in adult hippocampal neurogenesis^{79,80} whereas bFGF seems to be not.⁷⁷

Finally the question shall be discussed whether our prenatal irradiation protocol caused some accelerated postnatal and/or age-related cell loss in neurons that had been compromised by prenatal irradiation. Although this possibility cannot be completely ruled out, the unchanged ratios between the number of hippocampal granule cells and the number of hippocampal pyramidal cells as well as between the number of cerebellar granule cells and the number of cerebellar Purkinje cells in the 30-month-old animals do not support such postnatal and/or age-related effects. Accelerated postnatal loss of hippocampal neurons, which are under normal conditions not reduced in number during aging,^{37,81} has been observed only for hippocampal pyramidal cells but not for hippocampal granule cells after single prenatal low-dose X-irradiation.¹⁰ Furthermore, age-related loss of Purkinje cells has been well documented for the rodent cerebellum,^{82–85} whereas the number of cerebellar granule cells is not diminished during aging (Sturrock 1989, Rutten et al. 2003).^{82,85}

In summary, VLDRPP irradiation of rats from E13 to E16 with a dose rate of 0.7 mGy/min had severe effects on numbers of cells whose final progenitors underwent division during the selected period of irradiation (hippocampal pyramidal and cerebellar Purkinje cells). This suggests that the vulnerable period for these cells closely overlaps with the phase of division of their final progenitors. However, irradiation also affected cells whose final progenitors underwent division after the selected period of irradiation (hippocampal and cerebellar granule cells). In this case reductions in neuron numbers resulted most probably from at least three different mechanisms, i.e., reductions in the number of granule cell progenitors earlier in development, reductions in the amount of growth factors and/or mitogens, and the absence of trophic support from postsynaptic targets. The present data are relevant in the context of the increasing interest in effects of very low dose rate protracted irradiation on the developing and adult organism (Brenner et al. 2003), in that they demonstrate that VLDRPP irradiation of rats from E13 to E16 with a dose rate of 0.7 mGy/min does result in a severe decrease in total numbers of hippocampal and cerebellar neurons in the offspring brain. Therefore the prenatal irradiation protocol applied in the present study shares an important feature with chronic low daily dose gamma irradiation of adult animals and humans that can lead to adverse health consequences.^{86,87}

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APPENDIX

	DG	CA1-3	ML	PL	GL	WM
$\bar{S}$	15	15	17	17	17	17
Obj. 1	20×	20×	20×	—	20×	20×
$\sum S_{Sm}$	—	—	118	—	101	73
$\sum p_1$	—	—	153	—	125	109
$\sum p_t$	—	—	1,630	—	1,341	1,240
CE_{pSt}	—	—	0.018	—	0.029	0.023
Obj. 2	40×	40×	—	40×	100×	—
sla-x sla-y [μm]	200	200	—	400	400	—
a [μm^2]	612	1,224	—	15	15	—
h [μm]	10	10	—	15	15	—
d [μm]	5	5	—	5	5	—
$\bar{t}$ [μm]	40.6	42.1	47.5	47.9	46.9	47.5
$\sum CS_{mb/pm}$	15	25	—	30	26	—
$\sum CS_1$	25	33	—	37	31	—
$\sum CS_t$	126	312	—	347	307	—
$\sum n_{mb/pm}$	102	86	—	110	89	—
$\sum n_1$	164	112	—	125	105	—
$\sum n_t$	775	1,074	—	1,188	996	—
CE_{n-t}	0.039	0.032	—	0.031	0.033	—

Details of the stereologic estimation procedures. $\bar{S}$, average number of sections showing the corresponding brain region on systematically and randomly sampled series of sections spanning the entire left hippocampus or the left cerebellar half, respectively. DG, hippocampal dentate gyrus (and granule cells within this layer); CA1-3, hippocampal CA 1-3 field (and pyramidal cells within this layer); ML, cerebellar molecular layer; PL, cerebellar Purkinje cell layer (and cerebellar Purkinje cells); GL, cerebellar granule cell layer (and cerebellar granule cells); WM, cerebellar white matter. Obj. 1, objective used for identification of the boundaries of the layers (or for point counting, respectively); sla-x and sla-y; distance between the points for point counting in mutually orthogonal directions x and y (see Figure 1B in Schmitz and Hof³⁰); $\sum S_{Sm}$, average number of points counted in the corresponding layer within the first paramedian sagittal section of the left cerebellar half; $\sum p_1$, average number of points counted

in the corresponding layer within the section showing the largest total cerebellar projection area; $\overline{\sum p_i}$, average number of points counted in the corresponding layer within the entire cerebellum; $\overline{CE_{p\Sigma i}}$, average predicted coefficient of error of the estimated volumes using the prediction method described by Garcia-Finana et al.²⁷ Obj. 2, objective used for counting neurons; sln-x and sln-y ; distance between the unbiased virtual counting spaces in mutually orthogonal directions x and y ; a and h , base and height of the unbiased virtual counting spaces; d , depth within the section at which the unbiased virtual counting spaces were placed; $\bar{t}$, measured actual average section thickness after histologic processing; $\overline{\sum cs_{mbfpm}}$, average sum of unbiased virtual counting spaces used for counting neurons in the corresponding layer within the section through the mid body of the left hippocampus (or within the first paramedian sagittal section of the left cerebellar half, respectively); $\overline{\sum cs_1}$, average sum of unbiased virtual counting spaces used for counting neurons in the corresponding layer within the section showing the largest total area of the hippocampal cell layers (DG plus CA1-3) (or within the section showing the largest total cerebellar projection area, respectively). $\overline{\sum cs_i}$, average sum of unbiased virtual counting spaces used for counting neurons in the corresponding layer within the entire hippocampus (or within the entire cerebellum); $\overline{\sum n_{mbfpm}}$, average number of neurons counted in the corresponding layer within the section through the mid-body of the left hippocampus (or within the first paramedian sagittal section of the left cerebellar half, respectively); $\overline{\sum n_1}$, average number of neurons counted in the corresponding layer within the section showing the largest total area of the hippocampal cell layers (DG plus CA1-3) (or within the section showing the largest total cerebellar projection area, respectively). $\overline{\sum n_i}$, average number of neurons counted in the corresponding layer within the entire hippocampus (or within the entire cerebellum); $\overline{CE_{n-i}}$, average predicted coefficient of error of the estimated total numbers of neurons using the prediction method described by Schmitz²⁹ and Schmitz and Hof.

Figure S1. Ratios of individual results for male and female offsprings of a given dam. Analysis of rats subjected to prenatal irradiation (black diamonds) and prenatal sham-irradiation (open diamonds). Data were calculated as $[(\text{male} - \text{female}) / (\text{male} + \text{female})]$ and are shown as individual values and means per group (lines). Data were arranged in the same order as in Figure 3 and Figure 5. Hi-V_{GCL} , volume of the hippocampal granule cell layer. Hi-V_{PCL} , volume of the hippocampal pyramidal cell layer (CA1-3). Hi-N_{GC} , total number of hippocampal granule cells. Hi-D_{GC} , density of hippocampal granule cells. Hi-N_{PC} , total number of hippocampal pyramidal cells (CA1-3). Hi-D_{PC} , density of hippocampal pyramidal cells (CA1-3). Ce-V_{WM} , volume of the cerebellar white matter. Ce-V_{GCL} , volume of the cerebellar granule cell layer. Ce-V_{ML} , volume of the cerebellar molecular layer. Ce-N_{PC} , total number of cerebellar Purkinje cells. Ce-N_{GC} , total number of cerebellar granule cells. Ce-D_{GC} , density of cerebellar granule cells.

Chapter 3

The aging brain: fewer neurons could be better

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ABSTRACT

Molecular and cellular markers of age-related alterations in the brain vary significantly between different brain regions and between different types of neurons. In contrast to what had been thought for years, it has recently become clear that only specific types of neurons show an age-related loss of cells. Based on previous work we hypothesize that there is an interrelationship between two important processes in the aging brain: some types of neurons in the aging brain show an accumulation of unrepaired nuclear (n) nDNA damage since no cells are lost during aging. In contrast, other types of neurons show no accumulation of unrepaired nDNA damage since the cells with the greatest decline in nDNA repair capacity and the highest amount of nDNA damage are lost during aging. Most interestingly, the former types of neurons seem to correlate strongly with those types of neurons afflicted in age-related cognitive decline and in the selective neuronal vulnerability in Alzheimer's disease. Therefore, modulation of the nDNA damage response by stimulation of nDNA repair processes, or by elimination of neurons with a high amount of unrepaired nDNA damage in the aging brain, may lead to a functional improvement in networks of these types of neurons and to a better functioning of the aging brain in general. Ultimately, the implication of this strategy may lead to the prevention of Alzheimer's disease.

INTRODUCTION

Mammalian aging is an inevitable process associated with a general decline in physiological function, and with an increased incidence of degenerative diseases and cancer. Importantly, basic processes involved in aging of the central nervous system contribute to several prevalent neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease.¹ The aging brain is characterized by several age-related alterations. On the molecular and cellular level, an important aspect of the aging process of the brain is an increased production of free radicals and an accumulation of nuclear (n) and mitochondrial (mt) DNA damage.^{2,3} This is accompanied by an age-related decline in the amount of nDNA repair.³⁻⁵ On the functional level, the cognitive capacity of the aging brain declines gradually in the elderly individual.^{6,7} Neuropathological studies indicate that shrinkage of the brain, loss of neurons and the presence of specific intracellular and extracellular protein aggregates are common age-related findings.⁸ However, it has been shown over the last years that markers of these age-related alterations vary significantly between different brain regions, as well as between different types of neurons.^{4,5,9-12} As the brain consists of various types of neurons with different morphologic, physiologic and functional features, it is of great importance to investigate the aging brain in a cell type-specific manner. The number of studies assessing the regional variability and cell type-specific alterations in the aging brain, however, is very limited. In this article, several of these studies are reviewed. Based on this, a novel hypothesis concerning a better functioning of the aging brain in general is outlined. This hypothesis might also have implications for preventing the development of AD.

REGIONAL VARIABILITY OF DNA DAMAGE AND REPAIR IN THE AGING BRAIN

In recent quantitative studies, the rates of spontaneous nDNA repair and mtDNA synthesis were analyzed for various cell types of the aging rodent brain.^{4,13} The results of these studies indicated that the rate of mtDNA synthesis decreases in all investigated types of neurons during aging (Table 1, first column). However, a cell-type-specificity was found for the rate of spontaneous nDNA repair in the aging rodent brain. Purkinje cells in the cerebellum, mitral cells in the olfactory bulb, and the large neurons in the lateral vestibular nucleus did not show an age-related decline of the rate of spontaneous nDNA repair, whereas all other investigated types of projection neurons did (Table 1, second column). Importantly, age-related decline in the rate of nDNA repair may result in an age-related accumulation of unrepaired nDNA damage and thus in cellular dysfunction.¹⁴ Other studies have shown a regional variability in the amount of unrepaired nDNA damage in the aging rodent brain,⁹ and the patterns found were compatible with the cell-type-specific findings concerning the rate of nDNA repair in the studies of Schmitz et al.^{4,13} Specifically, the cerebellum exhibited a much lower amount of unrepaired nDNA damage than the neocortex and the hippocampus (Table 1, third column). Together these results clearly indicated a previously unknown regional variability in the processing of nDNA damage in the aging rodent brain.

In addition to the accumulation of unrepaired nDNA damage, aging is associated with an accumulation of mtDNA deletions.¹⁰ Interestingly, there are also region-specific differences in the amount of mtDNA deletions in the aging rodent brain.¹⁰ With particular respect to the human brain, certain brain regions such as the neocortex and the hippocampus exhibit large amounts of mtDNA deletions in aged individuals, whereas the cerebellum does not show this phenomenon (Table 1, fourth column).¹⁵⁻¹⁷

RESPONSES TO NDNA DAMAGE

The response to nDNA damage in eukaryotic cells can be divided into three components: recognition of nDNA damage, a period of damage assessment (enforced by checkpoints), and the activation and implementation of the appropriate response, i.e. nDNA repair or apoptosis.^{18,19} The mechanisms and proteins involved in nDNA damage recognition are poorly understood. Over the last years, however, several genes and proteins involved in these mechanisms have been identified. One of the identified proteins that seems to have a crucial role in sensing DNA damage is ATM (ataxia telangiectasia mutated²⁰). This protein is involved in the detection of nDNA double strand breaks. In the context of nDNA damage, checkpoints in damage assessment establish barriers to prevent the perpetuation of injured genomes.¹⁹ Thus, these checkpoints will allow the cell to attempt to repair the nDNA damage or to die by apoptosis. ATM, ATR (ataxia telangiectasia rad 3 related²¹) and DNA-PK (DNA-dependent protein kinases²²) seem to act as checkpoint sensors signaling to both cell-cycle and apoptosis machineries. Eventually, checkpoint activation can lead to p53 phosphorylation and activation which, subsequently, initiates cell cycle regulator proteins such as p21 and GADD45,¹⁹ or apoptotic activator proteins such as Bax and caspases.²³ The decision whether or not to die by apoptosis is regulated by numerous

'pro- and anti-apoptotic' proteins of which only a subset have been identified. Among the powerful anti-apoptotic proteins in the mammalian nervous system are the neurotrophins. These proteins bind to the TrkA-receptor on the cell membrane and activate the PI(3)K pathway (phospho-inositide 3-kinase²⁴). Among the pro-apoptotic proteins, C-jun, Dp5, Bax, Bcl-xl, CD95, MDM2 and PERP have been identified.¹⁹ The major function of apoptosis is to remove cells that would hamper optimal function of the organism as a whole. The apoptosis process is designed to rapidly remove cells from tissues, tagging them for phagocytosis and recycling their constituent molecules.²⁵ The role of the main effectors in the apoptotic machinery is played by the caspases.²⁴ Caspases activate other proteins of the apoptotic machinery by cleavage, uncouple the catalytic and nDNA binding domains of poly-ADP-ribose polymerase, and activate a DNase to fragment the nDNA molecules.²⁴ By contrast, if the cell 'chooses' to repair the nDNA damage, the machinery of nDNA repair is able to activate specific repair proteins according to the nature of the nDNA damage.^{19,26} Double strand breaks can be repaired by homologous recombination or non-homologous end joining, whereas single strand damages can be repaired by nucleotide excision repair, base excision repair or mismatch repair.²⁶ It should be noted that the mismatch repair pathway is connected to the cell cycle and therefore cannot be used by postmitotic cells such as neurons.

However, if nDNA repair mechanisms become insufficient to repair the damage during aging (due to decreased repair capacity or to an increased amount of nDNA damage), nDNA damage will remain unrepaired and will accumulate. This may result in cellular dysfunction.¹⁴ Interestingly, recent studies (not carried out on the nervous system) suggest that aging may also alter the apoptotic response to genotoxic stimuli. For example, Suh et al. showed that the apoptotic potential is dramatically reduced in aged murine hepatocytes after treatment with a genotoxic agent.²⁷ On the basis of this result, the authors speculated that age-related down-regulation of apoptosis might provide the mechanism of the well documented increase in nDNA damage and mutations with age. As Suh et al. further indicate,²⁷ it is highly tempting to speculate on the implications of these findings to the aging brain (although data obtained from cells, which can undergo mitosis, do not necessarily hold for postmitotic cells²⁸). In this vein, we hypothesize that some neurons in the aging brain do not respond appropriately to nDNA damage, undergoing neither nDNA repair nor apoptosis. Considering the data summarized in Table 1, this might indeed be the case.

REGIONAL VARIABILITY OF CELL LOSS IN THE AGING BRAIN

It has been assumed in the neuroscience community for a long time that aging is associated with a loss of neurons throughout all regions of the brain, and that this loss of neurons would explain the functional decline of the brain during aging.²⁹⁻³¹ Recent investigations, however, have provided convincing evidence that age-related loss of neurons in the mammalian brain clearly exhibits a regional and cell-type-specific variability (Table 1, fifth column).^{12,29,30,32} In this regard it should be noted that especially the application of state-of-the-art stereological methods has tremendously contributed to this shift in the knowledge concerning regional variability of neuronal loss in the aging brain.^{33,34} For example, Merrill et al. recently showed

that the numbers of pyramidal cells in the entorhinal cortex and hippocampus are conserved even in aged rodents with behavioural deficits.³⁵ As age-related cognitive decline of rodents is associated with disturbances of the neocortex and of the hippocampus, Merrill et al. concluded that age-related processes other than neuronal loss in the hippocampus are likely to cause age-related cognitive deficits in rodents.³⁵ Importantly, a meta-analysis of the current literature regarding cell-type-specific loss of neurons in the aging rodent brain indicated that specific types of neurons do show a substantial age-related loss of cells (Table 1; fifth column). For example, up to 30% of Purkinje cells in the human cerebellum are lost,³⁶ and also a substantial amount of the mitral cells in the olfactory bulb are lost during aging in the rodent brain.³⁷ Most interestingly, in the aging rodent brain there is a striking inverse correlation such that types of neurons showing no decline of the rate of spontaneous nDNA repair (with the implication of no increase in the amount of unrepaired nDNA damage) exhibit an age-related loss of cells, and types of neurons showing a decline in the rate of spontaneous nDNA repair do not show an age-related loss of cells (Table 1, second vs. fifth column).

CELL LOSS AS A POTENTIALLY PROTECTIVE MECHANISM IN THE AGING BRAIN

Based on this inverse correlation we hypothesize that – at least in the aging rodent brain – cell loss is the basis of the apparent lack both of age-related decline in the rate of spontaneous nDNA repair and of age-related accumulation of unrepaired nDNA damage in certain types of

Figure 1. Short overview on some interactions between several molecular events involved in brain aging (right part of the figure, i.e., right from the dotted line) and their interrelations with AD pathogenesis (left part of the figure). References for these interactions can abundantly be found in the literature.

Table 1.

Brain region	Type of neurons	1	2	3	4	5	6
Neocortex				+	+		
	All pyramidal cells	+	+			-	+
	Layer V pyramidal cells	+	+			-	+
Hippocampus*				+	+		
	Granule cells	+	+			-	+
	CA pyramidal cells	+	+			-	+
Olfactory bulb**							
	Mitral cells	+	-			+	-
Cerebellum				-	-		
	Purkinje cells	+	-			+	-

1: Age-related decline in mtDNA biosynthesis^{4,13}

2: Age-related decline in rate of nDNA repair^{4,13}

3: Age-related accumulation unrepaired nDNA damage⁹

4: Age-related accumulation of mtDNA deletions¹⁷

5: Age-related loss of cells^{4,13}

6: NFT formation in AD^{4,13}

* Note that CA1 neurons develop NFT along with CA4 neurones but CA2 neurones and to a certain extent CA3 neurons are relatively resistant to NFT formation. Additionally whilst granule cells in the dentate gyrus develop NFT, these are generally scattered and few in number.

** With respect to the mitral cells of the olfactory bulb (MC), one has to consider that conflicting data about NFT formation in these cells have been reported in the literature. Indeed in the paper by Kovacs et al.⁵⁶ an abundance of NFT formation in MC has been reported in AD. However, this stands in contrast to reports by Braak et al.⁵⁷ and Esiri et al.⁵⁸ which have reported lack of NFT in MC in AD. Moreover, in the paper of Kovacs et al.⁵⁶ NFT formation in MC has also been described in controls not suffering from AD. This seems hardly to be compatible with the Braak staging, in which NFT formation starts in the transentorhinal cortex, not in the olfactory bulb. Unfortunately, in the study by Kovacs et al.⁵⁶ the formation of NFT in MC has not been investigated in the context of overall NFT in the brain. In summary, the question about NFT formation in MC in AD needs further investigation.

neurons. Conversely, we hypothesize that the lack of age-related cell loss is connected to both age-related decline in the rate of spontaneous nDNA repair and to age-related accumulation of unrepaired nDNA damage in other types of neurons.

According to these hypotheses, neuronal dysfunction during aging is mainly connected to an accumulation of damaged macromolecules within certain specific types of neurons not having an appropriate cell death mechanism for highly damaged cells. Most importantly, age-related decline in cognitive function of rodents seems to be connected to the disturbed function of those types of neurons which do not show an age-related loss of cells (Merrill et al., 2001). This opens the possibility that the aging rodent brain does benefit from age-related loss of neurons. Recent studies have pointed to the fact that the pattern of cell loss in the aging human brain is very similar to the corresponding pattern in the aging rodent brain.⁴

It is important to realize that even a substantial amount of neuronal loss does not necessarily need to result in clinically detectable functional decline of certain brain regions or symptoms. For example, up to 60% of the dopaminergic neurons in the substantia nigra might be lost before overt symptoms appear in Parkinson's disease.³⁸ Furthermore, substantial loss of cerebellar Purkinje cells has been reported in AD,³⁹ but signs of cerebellar dysfunction are not early symptoms in AD.

Thus, the induction of an appropriate response to severe age-related nDNA damage, i.e., either effective repair of this nDNA damage or loss of the corresponding cells, might be a new strategy to prevent age-related functional decline of the human brain.

AGE-RELATED CELL LOSS, NDNA DAMAGE, AND THE SELECTIVE NEURONAL VULNERABILITY IN AD

Alzheimer's disease is a prevalent and devastating neurodegenerative disorder with a selective neuronal vulnerability of largely unknown origin.^{40,41} The neuropathological hallmarks of AD comprise β -amyloid plaques and neurofibrillary tangles (NFT), consisting mainly of hyperphosphorylated tau protein.⁴¹ Importantly, NFT formation in AD exhibits a certain selectivity, i.e., distinct cell types and brain regions such as the entorhinal cortex and the hippocampus are much more vulnerable than others.^{12,41} Several attempts have been made in the literature to explain this so-called selective neuronal vulnerability in AD,⁴²⁻⁴⁹ but these explanations have remained unsatisfactory. The major risk factor for AD is aging, and several recent studies have indicated that the formation of the AD hallmarks is associated with the same deleterious molecular events which occur during aging.^{44,50,51} Figure 1 gives an overview of some of the most important processes involved in aging (right part of the figure) and in AD (right and left part). Interestingly, the same neuronal cell types and neural circuits that are devastated through

degeneration in AD are particularly vulnerable to age-related molecular, biochemical and cellular alterations.¹² For example, one of the very first events in AD seems to be an abnormally high amount of free radicals in the affected cells, which could be an early event leading towards the formation of NFT.⁵² Furthermore, there is a co-localization of truncated tau and nDNA fragmentation in AD neurons.⁵³ Most interestingly, a meta-analysis of the literature indicated a striking correlation: the formation of NFT primarily occurs in those types of neurons which do not show an age-related cell loss (Table 1).⁴ For example, a significant number of Purkinje cells is lost in sporadic AD,³⁹ exceeding the loss of Purkinje cells during aging. It is also known that Purkinje cells do not show formation of NFT in AD. Thus, although events involved in the aging process are also involved in AD, the extent of alterations in AD exceeds the corresponding alterations in aging. As already mentioned, tangle-bearing neurons in AD show a high degree of nDNA fragmentation.^{53–55} In the literature, this is discussed as reflecting an extraordinarily high amount of unrepaired nDNA damage within these cells rather than a sign of apoptosis.⁵⁵ Furthermore, it has been speculated that the age-related accumulation of unrepaired nDNA damage could be one of the essential steps in the formation of NFT.^{53,54} Considering this speculation, it might be attractive to hypothesize that certain types of neurons are protected from the formation of NFT in AD by the physiological cell loss during aging. Obviously, the cell loss during aging should not be confused with the complex mechanism and pattern of pathological cell loss occurring in AD, which likely involves a combination of several processes including β -amyloid plaque toxicity and age-related regional and cell-type-specific loss of neurons.

Nevertheless, the induction of an appropriate response to nDNA damage in all types of neurons by removing damaged neurons during the aging process in the brain, might be an attractive new strategy to prevent the formation of NFT in AD. According to our new hypothesis, future interventions aimed at the prevention of NFT formation in AD and aimed at preservation of brain function during aging should focus on the induction of an appropriate nDNA damage response by a selective removal of those neurons in the aging brain which show the highest amount of free radicals and nDNA damage. Further research on the mechanisms of the nDNA damage response during aging of the brain will therefore be of great interest. Obviously, the cell loss during aging should not be confused with the complex mechanism and pattern of pathological cell loss occurring in AD, which likely involves a combination of several processes including β -amyloid plaque toxicity and age-related regional and cell-type-specific loss of neurons. Nevertheless, the induction of an appropriate response to nDNA damage in all types of neurons by removing damaged neurons during the aging process in the brain, might be an attractive new strategy to prevent the formation of NFT in AD. According to our new hypothesis, future interventions aimed at the prevention of NFT formation in AD and aimed at preservation of brain function during aging should focus on the induction of an appropriate nDNA damage response by a selective removal of those neurons in the aging brain which show the highest amount of free radicals and nDNA damage. Further research on the mechanisms of the nDNA damage response during aging of the brain will therefore be of great interest.

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Chapter 4

The aging brain: accumulation of DNA damage or neuron loss?

Submitted

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ABSTRACT

Age-related molecular and cellular alterations in the central nervous system are known to show selectivity for certain cell types and brain regions. Among them accumulation of unrepaired nuclear (n) DNA damage can lead to irreversible loss of genetic information content. In the present study on the aging mouse brain, we observed a substantial accumulation of unrepaired nDNA damage in hippocampal pyramidal and granule cells as well as in cerebellar granule cells but not in cerebellar Purkinje cells. The reverse pattern was found for age-related reductions in total number of neurons. Only the total number of cerebellar Purkinje cells was significantly reduced during aging whereas the total numbers of hippocampal pyramidal and granule cells as well as of cerebellar granule cells were not. This formerly unknown inverse relation between age-related accumulation of nDNA damage and age-related loss of neurons may reflect a fundamental process of aging in the central nervous system.

INTRODUCTION

Compelling evidence in the literature points to a central role of accumulated nuclear (n) DNA damage in the aging process of postmitotic cells such as neurons in the central nervous system (CNS).^{1,2} However, the aging process does not affect the CNS uniformly.³ Rather various brain regions and types of neurons differ substantially in the amount of accumulated nDNA damage during aging.⁴ For instance, it has been shown that nDNA damage accumulates to a higher amount in the hippocampus during aging than in the cerebellum.⁵ Furthermore, we have recently shown for the mouse brain that hippocampal pyramidal and granule cells were affected by an age-related decline in the amount of spontaneous nDNA repair whereas cerebellar Purkinje cells were not.⁵ Noticeably, other studies on mice have indicated an age-related reduction in the number of cerebellar Purkinje cells^{7,8} whereas no alterations in the numbers of hippocampal pyramidal and granule cells as well as in the number of cerebellar granule cells were found during aging.^{6,9} Furthermore, at least hippocampal pyramidal cells as well as cerebellar granule and Purkinje cells are not replenished with age by neuronal progenitor cells.^{10–12} Based on these findings we have hypothesized that neurons in the mammalian CNS show either age-related accumulation of nDNA damage, connected with age-related decline in the ability to properly repair nDNA damage, or an age-related reduction in number.⁴ The aim of the present study was to test this hypothesis for the aging mouse brain. For this aim, both the relative amount of nDNA single strand breaks and the total numbers of cells were measured for hippocampal pyramidal and granule cells as well as for cerebellar granule and Purkinje cells in adult and aged animals taken from the same cohort of mice aged under controlled conditions.

MATERIAL AND METHODS

Animals

A cohort of $n = 80$ male Han/NMRI mice was bred and housed under specified pathogen free conditions at the Institut für Versuchstierkunde, RWTH Aachen University, Aachen, Germany (four animals per cage, air-conditioned rooms, 20° C, 60% humidity, 12:12 hr light:dark cycle with artificial lights on at 6:00 a.m., *ad libitum* access to water and Altromin standard diet [Altromin, Lage, Germany]). All experiments and investigations were performed in accordance with German animal protection law. Of these mice, $n = 56$ were allowed to die spontaneously (control group; mean age 25.7 ± 3.29 months [mean $\pm$ standard deviation; SD]; median [50%] survival 26.1 months; maximum survival 30.3 months; data provided as requested for experimental aging research in ¹²) (Fig. 1). The remaining mice were sacrificed at 11.3 ± 0.8 months of age ($n = 12$; "adult mice"; 100% survival of the control group) as well as at 27.8 ± 0.3 months of age ($n = 12$; "aged mice"; 28% survival of the control group). Six adult and six aged mice were analyzed in a cell type specific manner *in situ* for the relative amount of nDNA single strand breaks with In Situ Nick Translation in combination with quantitative autoradiography.¹⁴ The remaining six adult and six aged mice were investigated with high-precision design-based stereology¹⁵⁻¹⁷ for total numbers of hippocampal pyramidal and granule cells as well as for cerebellar granule and Purkinje cells using the optical fractionator.¹⁵⁻¹⁷

Analysis of the relative amount of nDNA single strand breaks

$N = 6$ adult mice (mean age = 10.7 ± 0.5 months; mean $\pm$ SD) and $n = 6$ aged mice (mean age = 27.8 ± 0.3 months) were sacrificed by cervical dislocation. The brains were immediately dissected out and hemisected exactly in the midsagittal line. Right and left brain halves were embedded in Tissue-tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and quickly frozen using a solution of acetone/dry ice at -68° C. The right brain halves were cut into 10 μ m thick frontal sections including the hippocampus or the cerebellum on a cryostat (type HM 500 OM; Microm, Walldorf, Germany). The sections were mounted on glass slides (SuperFrost Plus, Menzel, Braunschweig, Germany) and stored at -70° C until further processing. The left brain halves were not used in the present study and were stored for future experiments.

The relative amount of nDNA single strand breaks was investigated by means of In Situ Nick Translation (ISNT), carried out using a slight modification of the protocol given by Maehara et al. [8]¹⁷ as described in Korr et al.¹⁴ Briefly, sections were incubated for 60 min at 37° C with a reaction solution containing 50 mM Tris-HCl-buffer (pH 7.5), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 200 U/ml of E.coli DNA polymerase I (endonuclease-free, Boehringer, Mannheim, Germany), 10 μ mol/ml each of dATP, dGTP, dCTP and dTTP (Boehringer) and 15 μ l [³H]dTTP/ml (1.03 MBq/ml; American Radiolabeled Chemicals Inc., St. Louis, MO). Incubation was carried out using special *in-situ* chambers consisting of small frames made of silicon (height = 1 mm; comparable to customary adapters for *in situ* PCR) and coverslips placed onto the slides. The reaction was terminated by washing the slides with 50 mM Tris-HCl buffer (pH 7.5). Afterwards the sections were Feulgen stained, dipped into diluted Ilford K2 emulsion (3 : 2 with distilled water) at 42° C, exposed for 5 days at 4° C and developed in

Amidol (4 min, 18° C). After poststaining with hematoxylin the autoradiographs were covered with a coverslip using Entellan (Merck, Darmstadt, Germany).

Evaluation of the autoradiographs was carried out with a light microscope (Olympus BH-2 equipped with a 100x oil Olympus UplanFl objective, NA = 1.30). For each animal, two randomly selected, non-adjacent coded autoradiographs showing the hippocampus as well as two autoradiographs showing the cerebellum were selected. On each section 50 randomly selected cell profiles of each of the following types of neurons were investigated regarding the number of autoradiographic silver grains over the nucleus: hippocampal pyramidal cells (area CA1-3), hippocampal granule cells (dentate gyrus), cerebellar granule cells, and cerebellar Purkinje cells. Counting of silver grains of all types of neurons was performed by the same person blinded for the age of the mice. Background was corrected by investigating autoradiographs which were prepared in an identical manner as described above except for the application of DNA polymerase I.¹⁴ Furthermore, data were corrected for nuclear size, allowing direct comparisons between different cell types.¹⁴

Analysis of total numbers of neurons

N = 6 adult (12-month-old) mice and n = 6 aged mice (mean age = 27.8 ± 0.3 months; mean ± SD) were anesthetized with chloral hydrate (10% aqueous solution, 0.005 ml/g body weight, intraperitoneally) and were sacrificed by intracardial perfusion fixation with formalin solutions as previously described.¹³ After opening the skulls, the heads of the animals were postfixed in formalin solution for 24 h at 4° C. Afterwards the brains were removed, hemisected exactly in the midsagittal line, and further fixed in formalin solution for 10 days at 4° C. Both brain halves were cryoprotected in sucrose solution (10%, 20% and finally 30% sucrose in 0.1 M Tri-HCl buffer, 2 × 12 h per solution; 4° C), embedded in Tissue-tek (Sakura Finetek) and quickly frozen. Both brain halves were stored at -70° C. The left brain halves were not used in the present study and were stored for future experiments.

The right brain halves were entirely cut into serial, 30 µm thick frontal sections on a cryostat (type HM 500 OMV, Microm). Every tenth section was collected, mounted on a glass slide (Superfrost Plus; Menzel), dried, defatted with Triton X-100 (0.025%, 20 min; Merck), and stained with cresyl violet (0.01%, 10 min). Slides were dehydrated and coverslipped using DePeX (Serva, Heidelberg, Germany). On all sections showing the hippocampus, the granule and pyramidal cell layers were delineated as recently described.¹⁸ On all sections showing the cerebellum, the granule cell layer (including the Purkinje cell layer) was delineated. Delineations were carried out using a stereology workstation, consisting of a modified light microscope (Olympus BX50; Olympus, Tokyo, Japan), Olympus UplanApo objectives (10×, NA = 0.40; 40×, oil, NA = 0.75; 100×, oil, NA = 1.35), motorized specimen stage for automatic sampling (Ludl, Hawthorne, NY, USA), electronic microcator (Ludl), CCD color video camera (Hitachi, Tokyo, Japan), PC (CoWoTec, Magdeburg, Germany) with framegrabber board, and stereology software (StereoInvestigator, MicroBrightField, Williston, VT, USA). Delineations were performed at a final magnification of ×360 (i.e., using the 10× objective). Total numbers of neurons were investigated with the optical fractionator.^{15-17,20} Quantifications of total numbers

of every type of neuron were performed by the same person blinded for the age of the mice. All hippocampal pyramidal and granule cells as well as all cerebellar granule and Purkinje cells whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the brain regions of interest were counted. Estimated total numbers of neurons were calculated from the numbers of counted neurons and the corresponding sampling probabilities. Details on the counting procedures are summarized in Table 1.

Statistical analysis

For each animal and type of neuron, the frequency distribution of the 100 obtained (uncorrected) numbers of autoradiographic silver grains was calculated, with bin width of one and the center of the first bin at zero. These frequency distributions were used to calculate age-specific, averaged frequency distributions of uncorrected numbers of autoradiographic silver grains for each type of neuron. For instance, the $n = 6$ adult mice showed the following frequencies of hippocampal pyramidal cells with a total of six autoradiographic silver grains over the nucleus: 14, 6, 16, 8, 10, 18.

Accordingly, the average frequency of hippocampal pyramidal cells in the brains from the adult mice with a total of six autoradiographic silver grains over the nucleus was 12. These averaged frequency distributions of the adult mice were compared with the corresponding averaged frequency distributions of the aged mice using the Kolmogorov-Smirnov two sample test. Comparing averaged frequency distributions considered the fact that the 95% critical value ($\alpha = 0.05$) for the Kolmogorov-Smirnov two sample test depends on the number of data points (which was 200 when comparing 2 groups with 100 data points each). Statistical significance was established at $p < 0.0125$, since four different types of neurons were investigated simultaneously. These calculations were performed using SPSS (Version 11.5.0 for Windows; SPSS, Chicago, IL). Furthermore, for each group of mice and investigated type of neuron, mean and standard error of the mean were calculated for the mean autoradiographic silver grain number per nucleus (corrected for background and nuclear size) and the estimated total number

Figure 1. Survival curve of the mice who died spontaneously (control group; $n = 56$). The dashed vertical line indicates 50% survival of the cohort (26.1 months). The solid vertical lines indicate the time points at which adult mice (mean age 11.3 months) and aged mice (mean age 27.8 months) were sacrificed for analysis.

Figure 2. Cumulated relative frequency (CRF) distributions of uncorrected numbers of autoradiographic silver grains from adult mice (green lines) and aged mice (red lines). Dotted lines, individual frequency distributions; solid lines, averaged frequency distributions. Hi-PC, hippocampal pyramidal cells; Hi-GC, hippocampal granule cells; Ce-GC, cerebellar granule cells; Ce-PC, cerebellar Purkinje cells

Table 1. Details of the stereologic analyses to estimate total numbers of neurons.

Type of neuron	Obj	B (μm²)	H (μm²)	D (μm)	t (μm)	Σ OD	Σ Q-	CEpred[n]
Hi-PC	100x	900	4	141	8.7	313	913	0.034
Hi-GC	100x	625	4	100	8.5	203	919	0.034
Ce-GC	100x	64	5	250	10.2	446	1.555	0.026
Ce-PC	40x	3,600	5	220	10.9	261	507	0.045

Hi-PC, hippocampal pyramidal cells (CA1–3); Hi-GC, hippocampal granule cells; Ce-GC; cerebellar granule cells; Ce-PC, cerebellar Purkinje cells. Obj., objective used; B and H, base and height of the unbiased virtual counting spaces; D, distance between the unbiased virtual counting spaces in mutually orthogonal directions x and y; t, measured actual average section thickness after histological processing; Σ OD, average sum of unbiased virtual counting spaces used; Σ Q-, average number of counted neurons; CE_{pred}[n], average predicted coefficient of error of the estimated total numbers of neurons using the prediction method.^{15,16}

of neurons. Comparisons between adult and aged animals were performed with two-tailed unpaired Student’s t test.

Statistical significance was established at p < 0.0125, since four different types of neurons were investigated simultaneously. These calculations were performed using GraphPad Prism (Version 4.00 for Windows, GraphPad Software, San Diego, CA).

RESULTS

Relative amount of nDNA single strand breaks

For hippocampal pyramidal and granule cells as well as for cerebellar granule cells – but not for cerebellar Purkinje cells – statistically significantly different averaged frequency distributions of uncorrected numbers of autoradiographic silver grains were found when comparing adult with aged mice (p < 0.001 for hippocampal pyramidal and granule cells as well as for cerebellar granule cells, p = 0.587 for cerebellar Purkinje cells) (Figure 2). The cumulated relative frequency distributions of uncorrected numbers of autoradiographic silver grains of hippocampal pyramidal and granule cells as well as of cerebellar granule cells from the aged mice were shifted to the right compared with the corresponding frequency distributions from the adult animals (Figure 2). This indicated that the aged mice showed on average higher numbers of uncorrected autoradiographic silver grains per nucleus for hippocampal pyramidal and granule cells as well as for cerebellar granule cells, but not for cerebellar Purkinje cells. Analysis of autoradiographic silver grains corrected for background and nuclear size resulted in the same finding. The mean corrected number of autoradiographic silver grains of hippocampal pyramidal and granule cells

Figure 3. (A–D) Autoradiographic silver grain counts corrected for background and nuclear size [5], representing the relative amount of unrepaired nDNA single strand breaks. (E–H) Total numbers of neurons (N) in the brain of adult and aged mice. (A, E), hippocampal pyramidal cells [Hi-PC]. (B, F) hippocampal granule cells [Hi-GC]. (C, G), cerebellar granule cells [Ce-GC]. (D, H) cerebellar Purkinje cells [Ce-PC]. Closed bars, adult animals; open bars, aged animals. Data are shown as mean $\pm$ S.E.M.. *, $p < 0.0125$; **, $p < 0.005$; ***, $p < 0.001$ (results from two-tailed unpaired Student's *t* test).

as well as of cerebellar granule cells was significantly higher in the aged mice than in the adult ones (+97.6% for hippocampal pyramidal cells in area CA1–3, $t = 3.141$, $p = 0.012$; +57.4% for hippocampal granule cells, $t = 3.804$, $p = 0.004$; and +65.3% for cerebellar granule cells, $t = 4.938$, $p < 0.001$; Figure 3A–C).

Importantly, cerebellar Purkinje cells showed the highest mean corrected number of autoradiographic silver grains of all investigated types of neurons but did not show differences in mean numbers between aged and adult mice ($t = 1.292$, $p = 0.225$; Figure 3D). In summary, the autoradiographic analysis with In Situ Nick Translation demonstrated that cerebellar Purkinje cells had the highest relative amount of nDNA single strand breaks of all investigated types of neurons. However, age-related accumulation of nDNA single strand breaks was observed for hippocampal pyramidal and granule cells as well as for cerebellar granule cells, but not for cerebellar Purkinje cells.

Total numbers of neurons

The mean total numbers of hippocampal pyramidal and granule cells as well as of cerebellar granule cells did not differ between adult and aged mice (hippocampal pyramidal cells: $t = 0.635$, $p = 0.539$, Figure 3E; hippocampal granule cells: $t = 0.568$, $p = 0.583$, Figure 3F; cerebellar granule cells: $t = 0.603$, $p = 0.560$, Figure 3G). In contrast, the mean total number of cerebellar Purkinje cells was significantly reduced in the aged mice compared with the adult ones (–25.2%, $t = 3.313$, $p = 0.009$, Figure 3H).

DISCUSSION

The results of the present study are the first description of cell-type-specific alterations in the relative amount of nDNA single strand breaks *in situ* in the mouse brain during aging. Furthermore, we have estimated the total numbers of four different types of neurons with high-precision design-based stereology (optical fractionator) and found that only cerebellar Purkinje cells were reduced in number during aging. These results are in line with various reports on cell type specific neuron loss in the rodent brain during aging (for details see ⁴) and they extend earlier reports on region-specific accumulation of nDNA damage in the rodent brain⁴ in a cell type specific manner. It should be mentioned that, based on the selection of aged mice at 28% survival of the control group, the results of the present study were neither biased by the "survival-of-the-fittest" effect (which can be observed at approximately 40% survival of a cohort; see, e.g., ⁶) nor by the "oldest-of-the-old" effect (which can be observed at approximately 10% survival of a cohort see, e.g., ²¹).

Interestingly, our group recently showed an age-related decline in the rate of spontaneous nDNA repair in hippocampal pyramidal and granule cells but not in cerebellar Purkinje cells (data of cerebellar granule cells were not unequivocal) by using quantitative autoradiography after injection of tritiated thymidine on the same animal model (i.e., same mouse strain, identical housing conditions at the same animal facility, almost identical survival curve).⁶ Altogether these data point to the existence of the following, so far unknown correlation between nDNA damage, nDNA repair and neuron loss in the aging mammalian brain: certain types of neurons (such as hippocampal pyramidal and granule cells as well as cerebellar granule cells) suffer from age-related decline in the ability to properly repair nDNA damage, resulting in accumulation of nDNA damage. However, these types of neurons are not reduced in number during aging. Other types of neurons (such as cerebellar Purkinje cells) are reduced in number during aging, and the remaining cells show no difference in the ability to properly repair nDNA damage compared with cells in young animals and, thus, no accumulation of nDNA damage.

Importantly, age-related accumulation of nDNA damage plays an important role in impaired protein synthesis, increased production of free radicals and the formation of protein aggregations during aging, ultimately leading to severe cellular dysfunction.^{2,22} Therefore, age-related accumulation of nDNA damage in selective types of neurons instead of proper repair of this damage or otherwise loss of the corresponding cells may play an important role in the neurobiology of the aging brain as well as in the well-known selective neurovulnerability in various neurodegenerative diseases.^{3,22} The correlation between nDNA damage, nDNA repair and neuron loss in the aging mammalian brain demonstrated in the present study leads to the hypothesis that selective types of neurons, such as cerebellar Purkinje cells, utilize certain mechanisms to eliminate cells with high amount of nDNA damage during aging whereas others do not. One might speculate that this occurs in connection with the fact that certain types of neurons (such as cerebellar Purkinje cells) show higher amounts of nDNA damage than other types of neurons (as demonstrated in Figure 3 by autoradiographic silver grains corrected for background and nuclear size); however, this speculation needs to be tested in future studies. Anyway it is important to note that in other organs than the brain, the apoptotic response to nDNA damage was recently

found to be downregulated during aging.²³ Accordingly it is tempting to speculate that distinct types of neurons are subjected to this downregulation of apoptotic response whereas other types of neurons are not. It will thus be important to identify cell-type-specific differences in the molecular machinery responsible for nDNA damage responses and to identify target molecules for modulating these responses. In summary, attenuation of age-related accumulation of nDNA damage in neurons may be reached through several strategies: diminishing age-related increase in the amount of nDNA damage by prevention of increased free radical production etc., enhancing the capacity to repair nDNA damage, or – when damage is beyond a certain threshold – by specifically eliminating neurons with high amount of unrepaired nDNA damage.

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Chapter 5

Antioxidants and Alzheimer's disease: from bench to bedside (and back again)

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ABSTRACT

Purpose of review: Accumulating evidence from both animal and human studies indicates a major role for oxidative damage in the pathogenesis of Alzheimer's disease (AD), occurring even before symptoms arise and both β -amyloid containing plaques and neurofibrillary tangles are formed. This raises the possibility to prevent or at least slow down the progression of AD by the use of antioxidants. In this review, we present recent studies on the association between oxidative stress and AD pathology, and on the efficacy of dietary, exogenous antioxidants to prevent or attenuate the progression of AD.

Recent findings: Recent prospective studies have indicated that dietary intake of several exogenous antioxidants is associated with a lower risk for AD. This suggests that people at risk for developing AD or being in the early phases of this disease may benefit from intervention with exogenous antioxidants. However, the clinical studies carried out so far do not provide the final answer to whether antioxidants are truly protective against AD.

Summary: There is compelling evidence that oxidative stress is involved in AD pathogenesis, and several lines of evidence indicate that administration of antioxidants may be useful in prevention and treatment of AD. However, further clinical studies based on larger cohorts studied over a longer period of time are needed to test this hypothesis. Furthermore, for the future one might expect balanced upregulation of both exogenous and endogenous antioxidants as one of the best treatment strategies for preventing or at least slowing down the progression of AD.

INTRODUCTION

Alzheimer's disease (AD) is a devastating neuropsychiatric disorder with a tremendous impact on the individual and on society in general. The disease is clinically manifested by a progressive dementia¹ with concomitant behavioral and social problems.² The neuropathologic hallmarks of AD are the formation of neuritic plaques containing β -amyloid, neurofibrillary tangles (NFT) mainly consisting of paired helical filaments, and neurodegeneration, showing a selective neuronal vulnerability of largely unknown origin.^{3*} Approximately 5% of all AD cases have an early onset and are familial, based on mutations of either presenilin 1 (PS-1), presenilin 2 (PS-2) and/or the β -amyloid precursor protein (APP).⁴ By contrast, approximately 95% of all AD cases represent the sporadic or late onset form of the disease, not showing mutations of PS-1, PS-2 and/or APP. Aging as well as the apolipoprotein E4 (ApoE4) genotype have been shown to be the major risk factors for sporadic AD.^{3*} Accumulating evidence indicates a major role of free radicals and oxidative stress in AD pathogenesis and pathophysiology.^{5*,6} Free radicals are highly reactive molecules either donating an electron to or extracting an electron from molecules. The most important free radicals are oxygen derivatives such as superoxide and hydrogen peroxide.^{7,8**} Antioxidants act by catalyzing the breakdown of oxidants or by scavenging oxidants with the aim to prevent damage to important molecules such as nucleic acids, proteins, lipids and sugars.^{7,8**} Oxidative stress can be defined as the imbalance between

Figure 1. Major sources of free radicals in Alzheimer's disease, the consequences of free radical damage, and the antioxidant defences against free radical attack. Generally, endogenous/enzymatic antioxidants catalyse the breakdown of free radical species, whereas nonenzymatic/exogenous antioxidants react preferentially with free radicals by donating electrons. Transition metal binding proteins prevent the interaction of transition metals with hydrogen peroxide and superoxide producing highly reactive hydroxyl radicals. Among the repair mechanisms, DNA repair and mitochondrial renewal are the most powerful.

oxidants and antioxidants in favor of oxidants. Neurons seem to be particularly vulnerable to oxidative stress due to the substantial amount of oxygen consumption by the brain, the low glutathione content and a high proportion of polyunsaturated fatty acids in neurons.^{5*} Administration of antioxidants to AD patients or patients at risk for developing AD has therefore been proposed and limitedly tested as a preventative and therapeutic strategy to combat AD pathology. Here, we review recent studies concerning the association between oxidative stress and AD pathology and the efficacy of antioxidants in prevention and slowing down the progression of AD. In addition, implications for future directions of research are discussed.

OXIDATIVE STRESS IN AD

A large body of evidence has indicated a major role of oxidative stress and oxidative damage in brains of AD patients, with the highest amount of oxidative damage in brain regions mostly affected by AD pathology such as hippocampus and cortex.^{5*,6,9,10} In this regard, both β -amyloid

containing plaques^{11*-13} and NFT^{14,15*} seem to be closely associated with markers of oxidative stress. So far, the exact interrelations between β -amyloid containing plaques and NFT on one hand and the formation of oxidative damage in brains of AD patients on the other hand have not been established.^{12,15*} However, a recent study has indicated that the increase in oxidative stress in vulnerable neurons precedes β -amyloid plaque and NFT formation.^{16**}

Among the markers of oxidative stress in brains of AD patients, oxidative damage to proteins has been observed in hippocampal tissue by elevated levels of carbonyls in nuclei of neurons and glial cells as well as in neuronal cytoplasm.¹⁷ Oxidative modifications of proteins in the presence of sugars may result in the formation of advanced glycation end products (AGEs). Several studies have indicated that AGEs are present in both NFT and senile plaques in AD.^{18,19} Furthermore, several studies have indicated oxidative damage to lipids in AD such as elevated levels of tran-4-hydroxy-2-hexenal (4-HNE) in cerebrospinal fluid (CSF),²⁰ acrolein in the amygdala and hippocampus/ parahippocampal gyrus,²¹ F2-isoprostanes in CSF,²² and F4-neuroprostanes in the cortex²³ (see also^{24*}). In addition, elevated levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) indicating oxidative damage to DNA have been observed in CSF of AD patients.²⁵ Oxidative damage to DNA in brains of AD patients might also be indicated by elevated levels of DNA strand breaks.²⁶

In addition to studies having investigated oxidative damage to brain tissue or CSF in AD, recent studies have focused on signs of oxidative stress in non-neuronal tissue in AD. Mecocci and co-workers showed that lymphocytes of AD patients exhibit elevated levels of 8-OHdG and decreased levels of antioxidative carotenoids as compared to healthy aged control subjects.^{27*} Besides this, McGrath and co-workers have investigated blood plasma of AD patients and have found elevated levels of 4-HNE but lower levels of ascorbate (vitamin C) compared to controls.²⁸

As already mentioned, aging and the ApoE4 genotype are the major risk factors for sporadic AD.^{3*} Interestingly, both are associated with increased oxidative stress to the central nervous system. Aging is known to cause an increased production and amount of reactive oxygen species, and elevated markers of oxidative damage have extensively been documented in aged cells and tissues.^{29-31*,32} Furthermore, Ramassamy and co-workers have recently shown that the ApoE4 genotype is associated with higher oxidative insults in specific brain regions of AD patients.^{33*} In this regard, Lauderback and co-workers suggest a role for apolipoprotein E in antioxidative modulation of β -amyloid toxicity, with the E4 allele being not as effective in this role as the other alleles of apolipoprotein E.³⁴

Several enzymatic and non-enzymatic antioxidants are present in biological tissue to prevent oxidative damage by free radicals and reactive oxygen species.^{7,8***,31*} Enzymatic antioxidants are produced endogenously and catalyze the breakdown of free radicals and intermediate molecules (reactive oxygen species) usually in the intracellular environment.^{8***} Among these enzymatic antioxidants, superoxide dismutases metabolize superoxide anions – mainly produced during oxidative phosphorylation in mitochondria – to hydrogen peroxide. Subsequently, hydrogen peroxide is enzymatically processed by catalase and glutathione peroxidase to water.

In the presence of iron and copper, however, hydrogen peroxide can also be processed via the Fenton reaction forming highly reactive hydroxyl radicals. Non-enzymatic antioxidants mainly act as scavenging agents in oxidative chain reactions.^{7,31*} They can be divided into lipid phase chain breaking antioxidants such as tocopherols (among which is α -tocopherol, vitamin E), carotenoids (among which is vitamin A), flavonoids and ubiquinone, and aqueous phase chain breaking antioxidants such as ascorbate, glutathione and uric acid^{8**}. Additionally, other non-enzymatic antioxidants act by preventing the interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide. These are the so-called metal binding proteins such as transferrin, ferritin, and lactoferrin.^{8**}

To fully address the consequences of oxidative stress in AD pathogenesis, it would be attractive to search for altered levels of antioxidants in the brain of AD patients. Among the few studies published so far on this topic, Karelson and co-workers have found that elevated levels of lipid peroxidation in the inferior temporal lobe in AD correlate positively with levels of superoxide dismutase and catalase, but correlate negatively with total antioxidant capacity as measured by *in vitro* inhibition of standard linoleic acid peroxidation.^{35*} These results suggest that there is an upregulation of endogenous antioxidants in affected regions in the AD brain to compensate for the increased amount of oxidants. This, however, is not sufficient to combat the increased amount of oxidants within these brain regions. In summary, there is compelling evidence that oxidative stress is involved in AD pathogenesis (see also ref. 36). Particularly, oxidative stress seems to play an important role in early phases of AD even before the formation of β -amyloid containing neuritic plaques and NFT, as well as before the appearance of clinical symptoms. This raises the general possibility to act on oxidative damage in AD by decreasing the amount of free radical formation as well as by scavenging free radicals before major damage occurs.

EXPERIMENTAL STUDIES ON ANTIOXIDANTS IN VIVO

Over the last years, many experimental studies have addressed the impact of increased levels of non-enzymatic/exogenous or enzymatic/endogenous antioxidants on oxidative damage to the central nervous system. Several rodent studies have indicated that dietary supplementation with exogenous antioxidants may decrease age-related lipid peroxidation in the cortex,³⁷ oxidative injury to synapses,³⁸ and protein and RNA oxidation.^{39*} Furthermore, dietary supplementation with exogenous antioxidants may prevent ischemia-related apoptosis in hippocampal neurons,^{40,41} and has been shown to attenuate ozone-induced memory deficits and lipid peroxidation in rats⁴² as well as cypermethrin-induced oxidative stress in rat brain and liver.⁴³ Other studies have assessed the effects of upregulation of endogenous antioxidants, and have shown that overexpression of human cytosolic copper-zinc superoxide dismutase (Cu/Zn SOD) in transgenic mice may attenuate age-dependent oxidative damage in the brainstem⁴⁴, 3-nitropropionic acid induced neurotoxicity⁴⁵, and may improve cognitive functioning in aged rodents⁴⁶. Another study has shown a rescue of homocystein induced endothelial dysfunction by overexpression of glutathione peroxidase in transgenic mice, which might be caused by improved NO-regulation.^{47*} Moreover, superoxide dismutase and catalase mimetics have been shown to prolong the mean life span of *Caenorhabditis elegans*.^{48**} However, one should keep

in mind that an excessive upregulation of certain endogenous antioxidants without concomitant increase of other endogenous antioxidants (e.g., increase of Cu/Zn SOD without increase of glutathione peroxidase) might also have deleterious effects.^{49,50}

The use of mice transgenic for mutated human APP, PS-1 and PS-2 has been shown to be a valuable tool for modeling several aspects of AD pathology.^{51*} For example, Matsuoka and co-workers have demonstrated that mice carrying mutant human APP and PS-1 develop β -amyloid deposits at 10–12 weeks of age and show several features of the human disease.^{52*} These mice have shown a strong association between fibrillar β -amyloid and oxidatively damaged proteins (by increased brain levels of 3-nitrotyrosine) and lipids (by increased brain levels of 4HNE). Noteworthy, the levels of 3-nitrotyrosine and 4HNE increased in relation to age-associated increase in fibrillar amyloid load.^{52*} Other mice transgenic for human APP and PS-1 mutations have exhibited elevated markers of brain lipid peroxidation even before the formation of β -amyloid containing plaques.^{25*,53**} However, only a limited number of studies have evaluated the effects of antioxidants in these AD models. A recent study by Lim and co-workers has shown that administration of the phenolic exogenous antioxidant curcumin to mice transgenic for mutant human APP developing β -amyloid plaques results in decreased oxidative brain damage and β -amyloid pathology.^{54***} Besides this, Pratico and co-workers have investigated transgenic mice overexpressing mutant human APP, fed with a diet enriched in aluminium.^{55*} Compared to non-transgenic littermates fed with the same diet, these authors have found an increase in brain isoprostane levels in the transgenic mice, correlated to increased β -amyloid levels and accelerated plaque deposition. Administration of vitamin E reversed this effect of aluminium, as demonstrated by a reduction of brain isoprostane levels in transgenic mice fed with both aluminium and vitamin E.^{55*} β -amyloid levels and plaque deposition were also reduced in these animals.

In summary, several lines of evidence indicate that administration of antioxidants may be useful in prevention and treatment of AD. In this respect, scavenging of free radicals by non-enzymatic/exogenous antioxidants seems to be the most practical approach. This is due to the fact that many different non-enzymatic/exogenous antioxidants without major side effects are available, and there is currently no therapeutic approach known to increase levels of enzymatic/endogenous antioxidants in humans. However, among the most effective approaches might be a balanced increase of both non-enzymatic/exogenous and enzymatic/endogenous antioxidants.

CLINICAL STUDIES ON ANTIOXIDANTS IN AD

The encouraging findings in the experimental models discussed above have stimulated studies on humans testing the effects of non-enzymatic/exogenous antioxidants in treating and preventing AD. Early studies on this topic carried out between 1989 to 1997 (reviewed in ^{56–58}) indeed provided preliminary evidence that antioxidants may have a protective effect against the development of AD. In a recent Cochrane review the outcome of all unconfounded, double blind, randomized trials carried out up to June 2000 has been analyzed, in which treatment with

vitamin E at any dose was compared with placebo for patients with Alzheimer's disease.^{59*} Only one study (published by Sano and co-workers⁶⁰) was identified as meeting the inclusion criteria for Cochrane analysis. In this study focussing on patients diagnosed as having mild to moderate AD, a slower progression of AD in patients using vitamin E has been shown as compared to patients not using this antioxidant.⁶⁰ In the corresponding Cochrane review it has been concluded that there is – albeit insufficient – evidence for an efficacy of vitamin E in the treatment of people with AD, justifying further studies on this topic.^{59*}

Since oxidative stress seems to be involved in the earliest phases of AD,^{26**} it is an attractive issue to focus therapeutic interventions on the early phases of this disease. Accordingly, very recently three studies on this topic have been published. First, Commenges and co-workers have investigated 1,367 initially non-demented french people above 65 years of age, and found – during a 5 year follow-up period – an inverse relation between intake of flavonoids and the risk of incident dementia (of which AD is one possible cause).^{61*} Second, Morris and co-workers have investigated the relationship between AD incidence and intake of the antioxidant nutrients, vitamin E, vitamin C, and beta-carotene (the so-called Chicago Health and Aging Project^{62**}). In this prospective, longitudinal study the authors have followed up 815 people of 65 years of age and older free of AD at baseline. On an average of 1.7 years after baseline, food frequency questionnaires were obtained, and incident AD cases as evaluated in clinical examinations according to standardized criteria were taken as main outcome measure. The results of this study indicate that only vitamin E from food, but not other antioxidants or vitamin E from supplements, may be associated with a reduced risk for AD.^{62**} Interestingly, the investigators observed this association only among individuals without ApoE4 genotype, suggesting that people with ApoE4 genotype may not be protected from AD by the use of vitamin E.^{62**} Third, Engelhart and co-workers have determined whether dietary intake of beta-carotene, flavonoids, vitamin C and vitamin E is related to the risk for AD in a large population based, prospective cohort study (the so-called Rotterdam study).^{63**} This study includes 5,396 participants who were at baseline at least 55 years old, non-institutionalized, dementia free and whose dietary assessment was reliable. Incidence of AD based on standardized criteria was the main outcome measure. The results, obtained after a mean follow-up of 6 years, indicate that high dietary intake of vitamins C and E was associated with a significantly lower risk for AD. This relation was even more pronounced in current smokers, for whom intake of beta-carotene and flavonoids was also preventative.^{63**} In contrast to the Chicago Health and Aging Project,^{62**} in the Rotterdam study the association between intake of antioxidants and AD risk did not vary between ApoE genotypes.^{63*} This difference between the outcome of the Chicago Health and Aging Project^{62**} and the Rotterdam study^{63**} might be due to the different sample sizes in these studies.

CONCLUSIONS AND FUTURE DIRECTIONS

There is compelling evidence that oxidative stress is involved in AD pathogenesis. Furthermore, several lines of evidence indicate that administration of antioxidants may be useful in prevention and treatment of AD. Recent prospective studies have indicated that dietary intake of several

exogenous antioxidants is associated with a lower risk for AD, suggesting that intervention with antioxidants might be beneficial in the early phases of AD or in people at risk for developing AD. However, the clinical studies carried out so far do not provide the final answer to whether antioxidants are truly protective against AD. Further intervention trials and observational investigations based on larger cohorts studied over a longer period of time are needed to test this hypothesis (see also ^{64*}). Nonetheless, the idea that non-enzymatic/exogenous antioxidants might have beneficial effects on AD pathogenesis makes sense, and it seems unlikely that diets with high antioxidant content would negatively affect brain aging.

In addition to further clinical studies, would it make sense to go back to the bench on this topic? To answer this question, one should consider that all clinical trials carried out so far have focussed only on administration of non-enzymatic/exogenous antioxidants. As already mentioned, however, a balanced upregulation of both exogenous and endogenous antioxidants might be the one of the best treatment strategies for preventing or at least slowing down the progression of AD. Thus, this strategy of a balanced upregulation of both exogenous and endogenous antioxidants is currently under study in our and other laboratories, using transgenic mice overexpressing human endogenous antioxidants and fed with diets containing high levels of exogenous antioxidants as experimental models. It is anticipated that the results of these experimental studies will have substantial impact on new clinical trials for preventing or at least slowing down the progression of AD in the near future.

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Chapter 6

Caloric restriction reduces nuclear DNA damage accumulation and age–related neuronal shrinkage in mouse hippocampus

Submitted

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ABSTRACT

Aging of the brain is characterized by increased oxidative stress and accumulation of damage to macromolecules. Selective types of neurons, as hippocampal pyramidal and granule cells, are preferentially affected during aging. Our previous work on the mouse brain showed a significant age-related accumulation of unrepaired nDNA damage in hippocampal pyramidal and granule cells. Furthermore, we hypothesized that long term transgenic upregulation of Cu/Zn superoxide dismutase (SOD1) and dietary restriction of calories combined with upregulation of exogenous antioxidants attenuate age-related alterations in the mouse hippocampus. To test this hypothesis the effects of a diet restricted in calories and enriched in antioxidants (CR diet), and the effects of upregulation of SOD1 were investigated on alterations in hippocampal volumes, numbers of hippocampal neurons and size of hippocampal neurons during aging as well as on accumulation of unrepaired nDNA damage in hippocampal neurons at high age. To this end, four cohorts of mice were generated depending on diet and genotype. Mice of these cohorts were sacrificed at 12 and 24 months of age. The hippocampus of these mice was investigated with design-based stereologic techniques to quantify volumes, numbers of neurons and size of neurons, and with In Situ Nick Translation to quantify the relative amount of unrepaired nDNA single strand breaks *in situ*. The results showed that CR diet extended mean and most probably also maximum lifespan, resulted in hippocampal volume loss and substantially attenuated age-related reductions in the size of hippocampal granule cells, while overexpression of normal SOD1 did not affect any investigated parameter. Most importantly, the CR diet led to substantial reductions in the amount of unrepaired nDNA damage in hippocampal neurons in 24-month-old mice. CR was thus a more effective strategy to attenuate age-related alterations in hippocampal neurons of the mouse brain than upregulation of SOD1.

INTRODUCTION

Aging of the mammalian central nervous system is characterized by increased oxidative stress and accumulation of damage to macromolecules eventually leading to neuronal dysfunction.¹⁻³ (see also Chapter 3 of this thesis). Evidence has been found that accumulation of nuclear (n) DNA damage plays a central role in age-related cellular dysfunction.^{3,4} During aging, relatively high levels of nDNA damage occur in promoter regions of genes with central roles in synaptic plasticity, vesicular transport and mitochondrial function in the human brain, at least in the cerebral cortex.⁴ Aging is known to particularly affect neurons forming hippocampal and cortical circuitries.⁵ Although there is no detectable loss of neurons in hippocampus or cerebral cortex during normal aging,³ neurons within these regions show high amounts of DNA damage^{3,6-8} and altered expression of genes involved in the response to DNA damage⁴ and in synaptic plasticity.^{5,9,10} During aging, accumulation of unrepaired nDNA damage and decline in the rate of nDNA repair occurs preferentially in cortical and hippocampal neurons (cf. Chapter 4 of this thesis).^{6,11,12} Accumulating evidence suggests that oxidative stress initiates age-related damage accumulation^{1,13,14} and the use of antioxidants has therefore been proposed as an effective strategy to prevent or attenuate age-related processes in the brain^{15,16} (cf. Chapter 5 of this thesis). Dietary means can be employed to upregulate the amount of exogenous antioxidants,

while transgenic approaches can be used to upregulate endogenous enzymatic antioxidants. Interestingly, transgenic expression of normal human Cu/Zn superoxide dismutase (SOD1), a main endogenous antioxidant scavenging superoxide anion, in mice has been shown to result in attenuation of age-related accumulation of oxidative damage to DNA and protein in the brain stem¹⁷ and can attenuate neurodegeneration after various forms of injury to the mammalian brain.¹⁸⁻²¹ However, direct proof that upregulation of endogenous antioxidants affects age-related alterations in hippocampus is currently lacking. Besides the use of antioxidants, various age-related alterations such as age-related DNA damage in the brain can be attenuated by restricting dietary intake of calories, i.e. caloric restriction (CR).^{22,23} CR is furthermore the only known strategy that prolongs mean and maximum life span in various animal species.^{24,25} Molecular studies to assess the effects of CR and antioxidants were previously carried out on homogenates of entire brains or entire brain regions of laboratory animals which does not allow for cell-type-specific analyses. As different brain regions and types of neuron are known to be differently affected by aging (as explained in detail in Chapters 3 and 4 of this thesis),^{3,5} studies carried out on homogenates cannot assess the exact effects of caloric restriction or antioxidants on age-related alterations in those types of neurons that are most affected during aging. Based on these considerations we hypothesized that a diet restricted in calories and enriched in antioxidants, as well as transgenic upregulation of human SOD1 influence age-related alterations in the mouse hippocampus. Four cohorts of mice were generated based on diet and genotype. Mice were sacrificed at 12 and 24 months of age and the brains of these mice were analyzed. The present study focused on the analysis of age-related alterations in hippocampal volumes, numbers of hippocampal neurons and size of hippocampal neurons with design-based stereologic techniques, and on alterations in levels of nDNA damage in hippocampal neurons at high age with In Situ Nick Translation, a molecular technique to quantify unrepaired nDNA single strands breaks *in situ*.²⁶

MATERIALS AND METHODS

Animals

Breeder pairs consisting of male mice transgenic for normal human SOD1²⁷ on a C57Bl6 background (SOD mice) and female wild type mice (C57Bl6) from the same colony were purchased from Jackson Laboratories (Bar Harbor, ME, USA; stock #002629, hemizygous C57BL/6-tgN(SOD1)3Cj); and transported to the Central Animal Facilities, Maastricht University, Maastricht, the Netherlands. SOD mice express three times the normal level of SOD1 in blood, brain, and fibroblasts.²⁷ Breeding procedures were carried out with hemizygous male SOD mice and wild type female mice for several generations. Offsprings were genotyped with standard PCR protocols. Breeding was performed until n=120 male hemizygous SOD mice and n=120 male wild type mice were available, with all of these mice born within a period of 12 weeks. After weaning on postnatal day 21 n=60 SOD mice and n=60 wild-type mice were fed with a control diet (described in the next paragraph; SOD-CD mice and WT-CD mice). The remaining n=60 SOD mice and n=60 wild-type mice were fed with a diet restricted in calories and enriched in antioxidants (also described in the next paragraph; SOD-CR mice and WT-CR mice). All mice were housed in individual cages under specified pathogen free conditions at the

Central Animal Facilities, Maastricht University, Maastricht, The Netherlands. All experiments were approved by the local Animal Ethical Committee and were performed in accordance with the dutch animal protection law.

Diets

The control diet was a derivation from the “N/N85” diet described by Weindruch and colleagues (1986)²⁸, and the diet restricted in calories and enriched in antioxidants a derivation from the “N/R50” diet described by these authors.²⁸ The N/R50 diet contained 50% less calories than a normal mouse diet while the N/N85 diet contained 85% of normal caloric intake for mice to ensure complete consumption of diet pellets and adequate control over caloric intake.²⁸ Both diets were custom made by Altromin (Lage, Germany). In the appendix of this study the composition of both diets is provided in detail.

The N/N85 diet was fed in portions of 3.0 to 3.2 grams on Mondays, Tuesdays, Wednesdays and Thursdays and in portions of 9.0 to 9.2 grams on Fridays according to Weindruch and colleagues (1986).²⁸ The N/R50 diet was fed in portions of 3.0 to 3.2 grams on Mondays and Wednesdays and in portions of 6.0 to 6.4 grams on Fridays according to these authors.²⁸

Time points of investigations

General health conditions and survival of the mice was inspected every other day. Body weight of the mice was recorded once per eight weeks during the entire life span of the mice. Mice were sacrificed and their brains analyzed at two time points (arrows in Figure 1A). At a mean age of 12 months, six mice per group were sacrificed by perfusion–fixation and their brains were used for immunohistochemical localization of human SOD1 as well as for design–based stereologic analysis of hippocampal volumes, numbers of hippocampal neurons in areas CA1–2 and dentate gyrus (DG), and mean size of hippocampal neurons in areas CA1–2 and DG. At a mean age of 24 months (when approximately 80% of the SOD–CD and WT–CD mice had spontaneously died), 11 mice per group were sacrificed. Six of these 11 mice per group were sacrificed by perfusion–fixation. The brains of these mice were processed and used for immunohistochemical localization of SOD1 as well as for design–based stereologic analyses of hippocampal volumes, numbers of neurons and mean neuronal size. The remaining five mice per group were sacrificed by cervical dislocation. The brains of these mice were processed and used for measurements of the relative amount of unrepaired nDNA single strand breaks (SSB) in hippocampal pyramidal and granule cells at 24 months of age.

Tissue processing for immunohisto–chemistry and stereology

A total of 48 mice (6 per group, 2 time points) were used for design–based stereologic analysis. These mice were sacrificed during intracardial perfusion–fixation with formalin solutions as previously described (cf. Chapter 4 of this thesis).²⁹ After opening the skulls, the heads of the animals were post–fixed in formalin solution for 24 h at 4° C. Afterwards the brains were removed, hemisected in the midsagittal line, and further fixed in formalin solution for 10 days

at 4° C. Left brain halves were cryoprotected in sucrose solution (10%, 20% and finally 30% sucrose in 0.1 M Tri-HCl buffer, 2×12 h per solution; 4° C), embedded in Tissue-tek® (Sakura Finetek), quickly frozen and stored at -70° C. Then they were entirely cut into serial, 30 µm thick frontal sections on a cryostat (type HM 500 OMV, Microm). The right brain halves were not used in the present study and were stored for future experiments.

Immunohistochemical detection of human SOD1 in hippocampus

One series of every tenth section through the hippocampus was collected for immuno-histochemical detection of SOD1 with anti human SOD1 antibody.³⁰ SOD1 immunohistochemistry was carried out in a free floating manner according to standard protocols as previously described.³¹ Briefly, avidin-biotin immunoperoxidase labeling was conducted by incubating sections overnight with primary antibody (polyclonal anti-human SOD1 antibody,^{30,32} 1:5,000) at 4° C after rinsing and blocking endogenous peroxidase activity with 0.3% H₂O₂ in 0.01M TBS. After incubation and rinsing with TBS and TBS-T, the sections were incubated in biotinylated secondary IgG antibodies (biotinylated donkey anti-rabbit IgG 1:800; Jackson, West Grove, PA, USA) for 1.5 hours at room temperature (RT), followed by ABC-kit (90 min at RT; Vectastain, Burlingame, CA, USA). The sections were then simultaneously incubated in 3,3'-Diaminobenzidine (Sigma, Uithoorn, The Netherlands) containing 0.03% H₂O₂ in TBS for 10 min at RT. Sections were rinsed with TBS, mounted on gelatinized glass slides, dehydrated, cleared and coverslipped using DePeX (Serva, Heidelberg, Germany). Immunoreactivity in hippocampal area CA 1-2, and dentate gyrus was photographed using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a 10x objective (Olympus), and a Hitachi HV-C20A camera (Hitachi Kokusai Electronics, Tokyo, Japan) connected to a computer which stored the acquired images. All images were acquired under identical conditions. Final figures were constructed using Corel Draw Version 11.0 (Corel, Ottawa, Canada) and Adobe Photoshop Version 7.0 (Adobe, San Jose, CA, USA). No adjustments of contrast and brightness were made.

Analysis of volume, numbers of neurons and mean size of neurons in the hippocampus

Another series of every tenth section through the hippocampus was collected, mounted on glass slides (Superfrost Plus; Menzel, Braunschweig, Germany), dried, defatted with Triton X-100 (0.025%, 20 min; Merck), and stained with cresyl violet (0.01%, 15 min). Slides were dehydrated and coverslipped using DePeX (Serva, Heidelberg, Germany). All stereologic procedures were carried out using a stereology workstation, consisting of a modified light microscope (Olympus BX50; Olympus, Tokyo, Japan), Olympus UplanApo objectives (10×, NA = 0.40; 100×, oil, NA = 1.35), motorized specimen stage for automatic sampling (Ludl, Hawthorne, NY, USA), electronic microcator (Ludl), CCD color video camera (Hitachi, Tokyo, Japan), PC (CoWoTec, Magdeburg, Germany) with framegrabber board and stereology software (StereoInvestigator, MicroBrightField, Williston, VT, USA) and 17" television screen monitor (Eizo, Nanao, Japan). Delineations for volume measurement of the entire hippocampus, hippocampal gray matter, hippocampal area CA 1-2 and area DG were performed according to descriptions in the literature³³ at a final magnification of ×360 (i.e., using the 10× objective). From the obtained data, the mean volumes of the entire hippocampus, hippocampal gray matter, hippocampal white

matter (defined as volume of the entire hippocampus minus volume occupied by gray matter), as well as of hippocampal area CA 1–2 and DG were calculated. Numbers of pyramidal cells in hippocampal area CA1–2, and granule cells in DG were estimated with the 100× oil objective and using the Optical Fractionator probe as previously described (cf. Chapters 2 and 4 of this thesis).^{34,35} Details on the stereological quantification of numbers are summarized in Table 1. Briefly, all pyramidal and granule cells whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the brain regions of interest were counted. Estimated numbers of neurons were calculated from the numbers of counted neurons and the corresponding sampling probabilities. The mean perikaryal size of pyramidal cells in hippocampal area CA 1–2 and granule cells in DG was estimated with the Nucleator probe³⁶ on all cells counted during the Optical Fractionator procedure.

Analysis of the relative amount of nuclear (n) DNA single strand breaks

Five 24-month-old mice per group were used for the analysis of the relative amount of nuclear (n) DNA single strand breaks (SSB). These mice were sacrificed by cervical dislocation, the brains were immediately dissected out and hemisected in the midsagittal line. Right brain halves were embedded in Tissue-tek® (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and quickly frozen using a solution of acetone/dry ice at -68° C. The right brain halves were cut into 10 µm thick sagittal sections including the hippocampus at a cryostat (type HM 500 OM; Microm, Walldorf, Germany). The sections were mounted on glass slides (SuperFrost Plus; Menzel) and stored at -70° C until further processing. Left brain halves were dissected into multiple regions and quickly frozen using liquid nitrogen. These left brain halves were stored at -70° C for future investigations. On sections of the right brain halves SSB was investigated by means of In Situ Nick Translation (ISNT). ISNT was carried out using a slight modification of the protocol given by Maehara et al.²⁶ as described in Korr et al.²⁹ Briefly, sections were incubated for 60 min at 37° C with a reaction solution containing 50 mM Tris-HCl-buffer (pH 7.5), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 200 U/ml of E.coli DNA polymerase I (endonuclease-free; Boehringer, Mannheim, Germany), 10 µmol/ml each of dATP, dGTP, dCTP and dTTP (Boehringer) and 15 µl [³H]dTTP/ml (1.03 MBq/ml; American Radiolabeled Chemicals Inc., St. Louis, MO). Incubation was carried out using special in-situ chambers consisting of small frames made of silicon (height = 1 mm; comparable to customary adapters for In Situ PCR) and coverslips placed onto the slides. The reaction was terminated by washing the slides with 50 mM Tris-HCl buffer (pH 7.5). Afterwards the sections were Feulgen-stained, dipped into diluted Ilford K2 emulsion (3:2 with distilled water) at 42° C, exposed 5 days at 4° C and developed in Amidol (4 min, 18° C). After poststaining with hematoxylin the autoradiographs were covered with a coverslip using Entellan® (Merck, Darmstadt, Germany). Evaluation of the autoradiographs was carried out with a light microscope (Olympus BH-2 equipped with a 100x oil Olympus UplanFl objective, NA = 1.30). For each mouse, one coded autoradiograph showing the hippocampus was selected. On each section 100 consecutive cell profiles of both pyramidal cells in hippocampal area CA1–2 and granule cells in DG were investigated regarding the number of silver grains over the nucleus. Background was corrected by investigating autoradiographs which were prepared in an identical manner as described above but omitting the application of DNA polymerase I.²⁹ Furthermore, data were corrected for nuclear size.²⁹ From the obtained data mean grain numbers per nucleus were calculated for each type of neuron and animal.

Statistical analysis

For each group of mice mean and standard error of the mean were calculated for all investigated parameters. The mean body weights of the mice were analyzed with non-linear regression analysis and extra-sum-of-squared F tests for nested models focusing on age-related changes in mean body weights per group, and with two-way analysis of variance at selected ages focusing on differences between groups due to diets and genotype. Data of hippocampal volumes, numbers of hippocampal neurons and mean size of hippocampal neurons were analyzed with multivariate analyses focusing on the effects of aging, diets and genotype. When significant differences were found, replicate means were compared with Bonferroni post hoc tests for pairwise comparison. Data of mean relative amounts of nDNA single strand breaks were analyzed with two-way analysis of variance. Statistical significance was established when *p* was found to be smaller than 0.05. All calculations were performed using SPSS (Version 12, SPSS Inc., Chicago, USA) and graphs were created using GraphPad Prism (Version 4.00 for Windows, GraphPad Software, San Diego, USA).

Table 1. Details of the stereologic analyses to estimate numbers of neurons. PC, pyramidal cells in hippocampal area CA1–2; GC, granule cells in hippocampal dentate gyrus (DG); Obj., objective used; B and H, base (μm^2) and height (μm^2) of the unbiased virtual counting spaces; D_x and D_y , distance between the unbiased virtual counting spaces in directions *x* (μm) and *y*, respectively; *t*, measured actual average section thickness after histological processing (μm ; ΣOD , average sum of unbiased virtual counting spaces used; ΣQ^- , average number of counted neurons; $CE_{pred}[n]$, average predicted coefficient of error of the estimated numbers of neurons using the prediction method^{35, 37}

Type of neuron	Obj.	B	H	D_x	D_y	<i>t</i>	ΣOD	ΣQ^-	$CE_{pred}[n]$
PC, area CA 1–2	100x	900	4	120	120	8.1	209	686	0.038
GC, area DG	100x	324	4	100	100	7.8	172	726	0.037

RESULTS

Increased SOD1 immunoreactivity throughout the hippocampus of SOD1 transgenic mice

The intensity of human SOD1 immunoreactivity was substantially increased in hippocampus of the SOD mice as compared to the wild-type mice (Figure 1). SOD1 immunoreactivity was detectable throughout the hippocampal neuropil and within the hippocampal pyramidal and granule cell layer of the dentate gyrus in the SOD mice (Figure 1 B, D, F and H). No differences in SOD1 immunoreactivity were observed between the SOD-CD mice and the SOD-CR mice.

Caloric restriction extended lifespan whereas overexpression of human SOD1 did not

Mean lifespan was 22.9 ± 4.7 months for the WT-CD mice and 21.9 ± 4.6 months for the SOD-CD mice, while maximum lifespan was 27.3 months for the WT-CD mice and 26.5 months for the SOD-CD mice. There were no differences between the survival curves of the WT-CD mice and the SOD-CD mice (Figure 2A). Determination of the mean and maximum lifespan of the

Figure 1. Immunohistochemical detection of human SOD1 in hippocampal area CA 1–2 (a to d) and dentate gyrus (e to h) of 12-month-old wild type mice fed with control diet N/N85 (a and e), 12-month-old mice transgenic for normal human SOD1 fed with control diet N/N85 (b and f), 12-month-old wild type fed with caloric-restricted/antioxidant-enriched diet N/R50 (c and g) and 12-month-old mice transgenic for normal human SOD1 fed with caloric-restricted/antioxidant-enriched diet N/R50 (d and h). Arrows indicate the hippocampal pyramidal cell layer in a to d and the granule cell layer in dentate gyrus in e to h. Note the increased SOD1 immunoreactivity throughout the gray and white matter of the hippocampus of the mice transgenic for normal human SOD1 (b, d, f, and h) compared to the wild type mice (a, c, e, and g). Scale bar represents 100 μ m.

Figure 2. Data of survival (a) and body weight (b) of wild-type mice fed either with diet N/N85 (WT-CD) or with diet N/R50 (WT-CR) as well as of SOD transgenic mice fed either with diet N/N85 (SOD-CD) or with diet N/R50 (SOD-CR). Data in (b) are given as mean (dots) and standard error of the mean (error bars) of the groups at intervals of three months. Closed dots, mean body weights of the WT mice; open dots, mean body weights of the SOD mice. Lines in (b) indicate the weight curves of the WT-CD, SOD-CD, WT-CR and SOD-CR mice according to second order polynomial regression analysis. Arrows above graph a indicate the two age points at which mice were taken from the cohorts for analysis. Data in (a) are from these mice of the cohorts which died spontaneously; data in (b) are from all mice.

WT-CR mice and the SOD-CR mice was not possible due to termination of the project at a mean age of the mice of 28 months. It should be noted that at 28 months of age, 56.8% of the WT-CR mice and the 78.8% of the SOD-CR mice were still alive, but 0% of the WT-CD mice and the SOD-CD mice.

Aging and caloric restriction influenced mean body weight

Multivariate analysis showed that aging significantly affected mean body weight in all groups of mice ($p < 0.001$; Figure 2B). Non-linear regression analysis and extra-sum-of-squared-F-tests for nested models indicated that second order polynomial regression analysis was significantly better at describing the weight curves in all groups of mice than first order polynomial regression analysis ($p < 0.001$ for all groups; see Table 2 for R^2 values of first order and second order polynomial regressions analysis).

Table 2. Details of non-linear regression analysis of the weight curves of wild-type mice fed either with the N/N85 diet (WT-CD) or the N/R50 diet (WT-CR) as well as of SOD transgenic mice fed either with the N/N85 diet (SOD-CD) or the N/R50 diet (SOD-CR) as a function of age. 1st, R^2 values of first order polynomial regression analysis ($Y = A + B \times X$); 2nd, R^2 values of second order polynomial regression analysis ($Y = A + B \times X + C \times X^2$); P, p values of extra-sum-of-squared F tests for nested models comparing first order polynomial regression analysis of the weight curves with second order polynomial regression analysis (null hypothesis: first order polynomial regression analysis describes the weight curves better than second order polynomial regression analysis). P values smaller than 0.05 are shown in bold face.

	WT-ND	SOD-ND	WT-CR	SOD-CR
1 st	0.249	0.279	0.050	0.080
2 nd	0.488	0.517	0.265	0.363
P	< 0.001	< 0.001	< 0.001	< 0.001

Table 3. P values of two-way analysis of variance of the effects of diet (D) and genotype (G) and their interaction (D*G) on the weight of the mice at different ages. P values smaller than 0.05 are depicted bold faced.

Age [months]	D	G	D*G
3	< 0.001	0.640	0.621
6	< 0.001	0.007	< 0.001
9	< 0.001	0.082	0.033
12	< 0.001	0.043	0.022
15	< 0.001	0.721	0.028
18	< 0.001	0.668	< 0.001
21	< 0.001	0.883	0.054
24	< 0.001	0.963	0.010

Furthermore, the diet had significant influence on the mean body weight of the mice at all investigated ages ($p < 0.001$ at all time points; Figure 1B; Table 3). In addition, small but statistically significant effects of the genotype of the mice on the body weight of the mice were found at 6 months of age ($p = 0.007$; Figure 1; Table 3) and 9 months of age ($p = 0.043$; Figure 1B; Table 3). The interaction between diet and genotype had significant effects on the body weight of the mice at 6, 9, 12, 15, 18, and 21 months of age (p values are given in Table 3).

Aging and caloric restriction reduced hippocampal volumes

Mean volumes of the entire hippocampus were significantly reduced as a result of aging ($p = 0.003$; -10.1%; Figure 3A) and diet ($p = 0.003$; -10.0%) but not as a result of the genotype of the animals (all p values of multivariate analysis summarized in Table 4). Aging and diet furthermore influenced the mean volumes of the hippocampal gray matter and the hippocampal white matter irrespective of the genotype of the mice ($p = 0.041$, -7.3% for age effect on hippocampal gray matter; $p = 0.002$, -10.7%, for age effect on hippocampal white matter; $p < 0.001$, -13.6% for diet effect on hippocampal grey matter; $p = 0.009$, -9.1%, for diet effect on hippocampal white matter; Figure 3B and 3C). Mean volumes of the hippocampal pyramidal cell layer CA 1–2 and hippocampal granule cell layer DG were significantly reduced as a result of diet ($p = 0.007$, -14.0%, for diet effects on hippocampal pyramidal cell layer CA 1–2; $p = 0.026$, -11.3%, for diet effects on hippocampal granule cell layer DG; Figure 3D and 3E) but not as a result of aging or the genotype of the mice. Post hoc tests showed that the age-related reductions in hippocampal volumes were not specific for a certain group of mice.

Aging, caloric restriction and transgenic overexpression of SOD1 did not affect numbers of hippocampal neurons

Numbers of hippocampal pyramidal cells in area CA 1–2 (Figure 3F) and granule cells in DG (Figure 3G) showed no alterations as result of aging, diet or genotype of the mice (p values are given in Table 4).

Aging and caloric restriction influenced the size of hippocampal neurons

The size of the pyramidal cells in area CA 1–2 was reduced as a result of diet ($p = 0.039$; -10.6%; Figure 3H), irrespective of age or the genotype of the mice. Post hoc tests showed that the diet-related reduction in size of hippocampal pyramidal cells in area CA 1–2 was not specific for any group of mice. Age affected the size of hippocampal granule cells in area DG ($p < 0.001$; -16.7%; Figure 3I) whereas diet or genotype did not. Post hoc tests showed that aging specifically resulted in reduced size of hippocampal granule cells in area DG in WT-CD mice and SOD-CD mice but not in WT-CR mice and SOD-CR mice.

Figure 3. Volume of the entire hippocampus (a), hippocampal gray matter (b), hippocampal white matter (c), hippocampal pyramidal cell layer area CA 1–2 (d) and hippocampal granule cell layer dentate gyrus (DG) (e), number of pyramidal cells in area CA 1–2 (f) and granule cells in area DG (g) as well as size of the pyramidal cells in area CA 1–2 (h) and granule cells in area DG (i) of wild-type mice fed either with diet N/N85 (WT-CD) or with diet N/R50 (WT-CR) as well as of SOD transgenic mice fed either with diet N/N85 (SOD-CD) or with diet N/R50 (SOD-CR). All graphs are divided into two parts by a vertical line for illustration purposes. Left parts of the graphs show mean and standard error of mean (SEM) of all investigated mice pooled for either age or diet. In case of Age closed bars represent 12-month-old mice and open bars 24-month-old mice; in case of Diet closed bars represent diet N/N85 and open bars diet N/R50. Right parts of the graphs show mean and SEM of the individual groups of mice. Here closed bars represent 12-month-old mice and open bars 24-months old mice. Data were analyzed with multivariate analysis, and differences were considered significant when $p < 0.05$. When significant differences were found in multivariate analysis, Bonferroni post-tests were performed to compare replicate means of 12-month-old mice versus 24-month-old mice per individual group. P values are provided as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) in the left parts of the graphs for significant differences in multivariate analysis and in the right parts of the graphs for significant differences in Bonferroni post-tests; all p values for multivariate analysis are given in Table 4.

Table 4. *P* values of multivariate analysis on the effects of age (A), diet (D), genotype (G) and the combination of these factors on the parameters analyzed in the present study. I, Volume of hippocampus; II, Volume of hippocampal gray matter; III Volume of hippocampal white matter; IV, Volume of cell layer CA1–2; V Volume of granule cell layer DG; VI, number of pyramidal cells in area CA1–2; VII, Number of granule cells in DG; VIII, Mean size of pyramidal cells in area CA1–2; IX, Mean size of granule cells in DG; X, relative amount of unrepaired nDNA single strand breaks in hippocampal pyramidal cells in area CA 1–2; XI, relative amount of unrepaired nDNA single strand breaks in hippocampal granule cells in DG. *P* values smaller than 0.05 are depicted bold faced.

	A	D	G	A*D	A*G	D*G	A*D*G
I	0.003	0.003	0.088	0.434	0.673	0.633	0.828
II	0.041	<0.001	0.125	0.826	0.89	0.692	0.713
III	0.002	0.009	0.103	0.316	0.585	0.493	0.715
IV	0.779	0.007	0.193	0.554	0.697	0.524	0.300
V	0.174	0.026	0.518	0.534	0.751	0.684	0.907
VI	0.539	0.925	0.264	0.948	0.583	0.863	0.985
VII	0.082	0.274	0.808	0.289	0.245	0.913	0.649
VIII	0.063	0.039	0.362	0.947	0.455	0.569	0.181
IX	<0.001	0.107	0.061	0.339	0.37	0.256	0.604
X		0.005	0.771			0.3	
XI		<0.001	0.835			0.453	

Caloric restriction – but not overexpression of human SOD1 – reduced the relative amount of unrepaired nDNA single strand breaks

The relative amount of unrepaired nDNA single strand breaks in hippocampal pyramidal cells in area CA 1–2 was significantly reduced as a result of diet ($p = 0.005$; -63.0%; Figure 4A) but not as a result of genotype. Diet furthermore reduced the relative amount of unrepaired nDNA single strand breaks in hippocampal granule cells in area DG ($p < 0.001$; -61.2%; Figure 4B) while genotype did not (all *p* values are given in Table 4).

DISCUSSION

In the present study we have shown that feeding mice with a certain diet restricted in calories and enriched in antioxidants (CR) resulted in substantial weight loss and extension of mean and maximum life span. We have furthermore shown that human SOD1 was expressed in the hippocampus of mice transgenic for human SOD1; an important prerequisite for testing effects of SOD1 on hippocampal neurons. The present study is the first high-precision stereologic study focusing on the effects of aging, caloric restriction (with a concomitant enrichment of antioxidants) and overexpression of SOD1 on the volumes of hippocampal regions, numbers of hippocampal neurons and their size in mice. We have shown that aging was associated with a reduced size of hippocampal granule cells and that caloric restriction attenuated age-related shrinkage of granule cells in mice. We have furthermore shown that CR was associated with

Figure 4. Relative amount of unrepaired nDNA single strand breaks (expressed as silver grains found in the analysis of autoradiographs) in hippocampal pyramidal cells in area CA 1–2 (a) and hippocampal granule cells in area DG (b) of wild-type mice fed either with diet N/N85 (WT–CD) or with diet N/R50 (WT–CR) as well as of SOD transgenic mice fed either with diet N/N85 (SOD–CD) or with diet N/R50 (SOD–CR) at 24 months of age. All graphs are divided into two parts by a vertical line for illustration purposes. Left parts of the graphs show mean and standard error of mean (SEM) of all investigated mice pooled for diet; right parts of the graphs show mean and SEM of the individual groups of mice. Closed bars represent diet N/N85 and open bars diet N/R50. Data were analyzed with multivariate analysis, and differences were considered significant when $p < 0.05$. When significant differences were found in multivariate analysis, Bonferroni post-tests were performed to compare replicate means of wild-type mice versus SOD mice. P values are provided as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) in the left parts of the graphs for significant differences in multivariate analysis and in the right parts of the graphs for significant differences in Bonferroni post-tests; all p values for multivariate analysis are given in Table 4.

hippocampal volume loss without concomitant loss of neurons. Moreover, our results show that CR substantially reduced the relative amount of unrepaired nDNA single strand breaks in hippocampal neurons of 24-month-old mice *in vivo*, whereas overexpression of human SOD1 did not.

Caloric restriction but not overexpression of SOD1 increased mean and maximum lifespan of mice

The finding that transgenic overexpression of SOD1 did not change mean or maximum lifespan of mice fed with control diet N/N85 implies that the level of SOD1 did not control lifespan in mice fed with this diet. Although exact data on mean and maximum life span of the WT–CR and the SOD–CR mice could not be established in the present study, the finding that 56.8% of the WT–CR mice and 78.8% of the SOD–CR mice were still alive at an age where all WT–CD mice and SOD–CD mice had died, indicates that mean and maximum life span were significantly increased as a result of CR. From an age of 18 months on, survival of the SOD–CR mice was always higher (at least up to 28 months of age) than survival of the WT–CR mice, suggesting that the combination of transgenic overexpression of SOD1 with CR had additive effects on survival of mice. It will be interesting to readdress this issue in future studies to establish mean and maximum life span of WT–CR mice and SOD–CR mice.

Age- and diet- related changes in body weight of mice

The mean body weight of the mice subjected to CR was significantly reduced during the entire life span (on average approximately 53%) as compared with the mice fed with the control diet. This finding is fully in line with several reports in the literature.²⁸ In contrast to this diet effect, we observed that overexpression of human SOD1 did not substantially affect the body weight of mice. Regression analysis furthermore showed that the weight curves of all groups of mice were best described with a second order polynomial regression curve, indicating that normal age-related regulation of body weight was not affected by CR or overexpression of human SOD1.

Aging and caloric restriction reduced hippocampal volumes in mice

The volumes of the entire hippocampus, hippocampal gray matter and hippocampal white matter of the investigated mice were significantly reduced as a result of aging. This finding, however, was only observed when a large number of animals was analyzed ($n = 48$ in this study), and was not specifically observed in a certain individual group of mice. These results are in line with several studies describing age-related hippocampal volume loss in large cohorts of humans,^{38,39} although the authors of another study did not observe significant age-related hippocampal volume loss in humans.⁴⁰

Our findings are the first showing that CR reduced the volume of the mouse hippocampus by approximately 10% (with 13.5% reduction in hippocampal gray matter and 9.1% reduction in hippocampal white matter). These findings are in line with a recent report of Markowska and colleagues describing that the volume of the putamen in rhesus monkeys under caloric restriction was approximately 16% smaller than in control monkeys.⁴¹ Although further research on the mechanisms underlying CR-induced volume loss is highly warranted, one could speculate that CR-induced stress and metabolic changes may necessitate strict control of the size of organs and brain regions. Although we did not measure brain weight and compared brain weight with body weight, it is noteworthy that the hippocampal volume of the WT-CR mice and SOD-CR mice was reduced by only 10% despite a 47% reduction in body weight, suggesting that brain weight and brain size are relatively conserved under CR conditions in mice.

Aging, caloric restriction and overexpression of SOD1 did not result in hippocampal neuron loss in mice

The results of the present study showed that overexpression of human SOD1 in mice did not alter numbers of hippocampal pyramidal cells in area CA1–2 and granule cells in DG. Moreover, our findings showed that aging did not change numbers of hippocampal pyramidal and granule cells, which is in line with several other studies in the literature on both rodents^{42,43} and humans^{44,45}. This finding furthermore confirmed that mechanisms responsible for age-related dysfunction of hippocampal neurons involve alterations at the subcellular level rather than mere loss of neurons.

Our finding that CR did not alter numbers of pyramidal cells in hippocampal area CA1–2 and granule cells in the dentate gyrus are – to the best of our knowledge – the first indicating a preserved number of hippocampal neurons in mice subjected to CR. Previous studies have assessed the effects of caloric restriction on the retina in the rat. In this respect, Obin and colleagues observed that caloric restriction increased light-dependent photoreceptor cell loss during aging in rats, as measured by photoreceptor cell density analysis.⁴⁶ On the other hand, Neufeld and Gachie recently showed that caloric restriction attenuated normal age-dependent loss of retinal ganglion cells as measured by density analyses.⁴⁷ Because normal aging in the retina seems to be connected to loss of neurons while normal aging of the hippocampus is not, it is difficult to compare potential mechanisms underlying age-related and diet-related changes in numbers of neurons in the aging retina with those in the aging hippocampus. Moreover, the study by Obin and colleagues⁴⁶ as well as the study by Neufeld and Gachie⁴⁷ were carried out with cell density measurements. In this respect, our recent study (cf. Chapter 2 of this thesis) indicated that quantitative analyses of cell densities may not be indicative of alterations in cell densities.⁴⁸ Analyses of neuronal densities were furthermore shown to result into incorrect data collection leading to false interpretation and conclusions, especially when the volume of the region of analysis was altered.⁴⁸ The use of modern, design-based stereologic techniques (as used in the present study) allows however high precision quantification of numbers of neurons free of bias.⁴⁹ Thus, the results of the studies by Obin and colleagues⁴⁶ and Neufeld and Gachie⁴⁷ may have been subject to bias, and it remains unclear whether caloric restriction attenuates or induces potential age-related loss of photoreceptor and ganglion cells in the retina.

The CR-induced volume loss observed in the present study was, most importantly, thus not accompanied by a loss of hippocampal neurons. Notably, CR-induced volume loss was largest in the hippocampal area CA1–2, and the pyramidal cells in CA1–2 showed a reduction in size as a result of CR. Therefore, our data argue that CR-induced volume loss of hippocampal area CA1–2 depended on reduced size of the pyramidal cells in this area. One could speculate that the reduced neuronal size of pyramidal cells in CR mice potentially reflected reduced cellular metabolism.

Age-related reduction in size of hippocampal granule cells in mice was attenuated by caloric restriction

In the present study we observed an age-related reduction (about 16%) in the size of hippocampal granule cells during aging. The dendritic tree of hippocampal granule cells is located in the hippocampal molecular layer where afferent axons from the entorhinal cortex terminate. Interestingly, the hippocampal molecular layer has been described as a hot spot in the aging brain⁵ as it is preferentially affected by several age-related alterations such as reduced expression of the presynaptic marker synaptophysin,¹⁰ reduced expression of the NR-1 subunit of AMPA and NMDA receptors,⁵⁰ reduced expression of Arc-1⁵¹ (an immediate early gene which has been correlated with spike activity) and a reduced number of axospinous and axosomal synapses.^{52,53} One might therefore speculate that the observed age-related reduction in size of the granule cells reflects or is associated with some of these age-related alterations. Interestingly, the reduced size of the hippocampal granule cells during aging was only observed in CD mice

but not in CR mice, suggesting that CR attenuated the normal age-related reduction in mean size of hippocampal granule cells in mice.

Caloric restriction reduced nDNA damage in hippocampal pyramidal and granule cells in mice

The relative amount of unrepaired nDNA single strand breaks in hippocampal pyramidal cells and in hippocampal granule cells was approximately 60% lower in the CR mice than in the CD mice. Previous studies have shown that CR reduced age-related increase of 8-OHdG levels²² and age-related mitochondrial damage in brain.^{54,55} Our findings are – to the best of our knowledge – the first showing that CR reduced the amount of unrepaired nDNA damage in hippocampal neurons. Attenuation of age-related accumulation of nDNA damage in neurons has been proposed to be achieved through several strategies:³ diminishing age-related increase in the amount of nDNA damage by prevention of increased free radical production etc., enhancing the capacity to repair nDNA damage, or – when damage is beyond a certain threshold – by specifically eliminating neurons with high amount of unrepaired nDNA damage (cf Chapter 3 and 4 of this thesis). In this respect, the preserved neuronal numbers in CR mice in the present study indicate that caloric restriction did not reduce nDNA damage in hippocampal neurons by eliminating neurons with high amounts of nDNA damage. Caloric restriction has been reported to reduce production of free radicals¹ and to increase the activities of nDNA repair enzymes⁵⁶ as well as to normalize the age-related decline in the rate of nDNA repair.⁵⁷ On this basis one can argue that CR reduced nDNA damage in hippocampal neurons by attenuation of oxidative stress and by enhancement of nDNA repair. Future studies will be employed to test these hypotheses.

Methodologic consideration: volume loss of a certain brain region does not necessarily indicate neuron loss in this brain region

Volume measurements of the present study indicated significant age-related and diet-related reductions in hippocampal volume. For instance, CR resulted in a 10% volume reduction of the hippocampus with an approximately 17% volume reduction in hippocampal cell layers. Importantly, the observed age-related and diet-related volume reductions were not associated with loss of neurons. Both aging and CR resulted however in significant cell-type-specific reductions in the mean perikaryal size of neurons. Thus, the observed loss of hippocampal volumes reflected reduced mean neuronal size and not a loss of neurons. This is a relevant observation as hippocampal volume loss in imaging studies is often interpreted as a sign of neurodegeneration and/or neuron loss.^{58,59} For example, Driscoll and colleagues recently reported that impaired performance in hippocampal-dependent tasks of humans was correlated with hippocampal volume loss.⁵⁹ Together with their observation of decreased hippocampal levels of *N*-acetyl-aspartate and creatine (i.e. neuronal metabolites detectable with non-invasive proton magnetic resonance spectroscopy) these authors interpreted their findings as implying neuronal loss and/or decreased neuronal density in the hippocampus during aging.⁵⁹ Our findings show that this interpretation might be incorrect and advocate caution when interpreting results of imaging studies towards cellular processes.

In summary, long term CR extended mean and most probably also maximum lifespan of mice, resulted in hippocampal volume loss and abolished age-related reductions in the size of hippocampal granule cells, while overexpression of normal SOD1 did not affect any investigated parameter. Most importantly, CR resulted in reduced levels of unrepaired nDNA damage in hippocampal neurons in aged mice. CR was thus a more effective strategy to attenuate age-related alterations in hippocampal neurons of the mouse brain than transgenic upregulation of SOD1.

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Ingredient	N/N85	N/R50	unit
Raw protein	173,194.0	301766.0	mg/kg
Raw fat	135,672.8	135889.8	mg/kg
Raw fiber	55,601.9	39659.5	mg/kg
Raw ashe	55,215.5	100075.3	mg/kg
Moisture	59,851.5	66271.0	mg/kg
Dissaccharide	271,002.0	182736.8	mg/kg
Polysaccharide	240,208.0	143495.0	mg/kg
Usable energy	4,024.1	3855.0	kcal/kg
Lysine	17,447.2	30345.1	mg/kg
Methionine	11,176.0	19897.9	mg/kg
Cystine	3,140.0	5495.0	mg/kg
Threonine	7,142.0	12507.3	mg/kg
Tryptophan	1,980.4	3467.7	mg/kg
Arginine	9,785.6	17133.4	mg/kg
Histidine	5,237.6	9169.6	mg/kg
Isoleucine	7,212.0	12630.2	mg/kg
Leucine	14,566.0	25502.1	mg/kg
Phenylalanine	7,116.8	12462.1	mg/kg
Valine	3,267.6	5728.1	mg/kg
Alanine	2,414.4	4235.1	mg/kg
Asparagine	3,545.6	6219.0	mg/kg
Glutamine	23,428.8	41026.1	mg/kg
Glycine	3,106.4	5443.8	mg/kg
Proline	12,580.4	22022.1	mg/kg
Serine	3,215.2	9133.1	mg/kg
Tyrosine	9,228.0	16154.8	mg/kg
Vitamin A	17,250.0	31650.0	IE/kg
Vitamin D3	575.0	1055.0	IE/kg
Vitamin E	230.5	374.8	mg/kg
Vitamin K3	11.5	21.1	mg/kg
Vitamin B1	23.0	42.3	mg/kg
Vitamin B2	23.3	42.8	mg/kg
Vitamin B6	17.3	31.7	mg/kg
Vitamin B12	0.05	0.08	mg/kg
Nicotinic acid	57.7	105.8	mg/kg
Panothenic acid	57.6	105.7	mg/kg
Folic acid	11.5	21.1	mg/kg
Biotin	0.23	0.40	mg/kg
Choline chloride	1,161.5	2,130.1	mg/kg
p-aminobenzoic acid	115.0	211.0	mg/kg
Inositol	126.0	230.3	mg/kg
Vitamin C	23.0	42.2	mg/kg
Calcium	9,388.2	16,839.0	mg/kg
Phosphorus	7,361.0	13,596.3	mg/kg
Magnesium	709.4	1,217.3	mg/kg
Sodium	2,476.0	4,437.0	mg/kg
Potassium	7,245.9	13,197.0	mg/kg
Sulfur	2,040.0	3,590.7	mg/kg
Chloride	3,640.8	6,675.0	mg/kg
Iron	179.1	326.4	mg/kg
Manganese	101.0	184.8	mg/kg
Zinc	69.6	133.0	mg/kg
Copper	5.6	10.1	mg/kg
Iodide	0.46	0.85	mg/kg
Molybdenium	0.20	0.36	mg/kg
Fluor	4.2	7.7	mg/kg
Selenium	0.27	0.44	mg/kg
Cobalt	0.14	0.27	mg/kg
Caproic acid	135.0	135.0	mg/kg
Lauric Acid	135.0	135.0	mg/kg
Myristine	135.0	135.0	mg/kg
Pentadecane	135.0	135.0	mg/kg
Palmitinic acid	16,470.0	16,470.0	mg/kg
Palmitoline	135.0	135.0	mg/kg
Margarine	135.0	135.0	mg/kg
Stearic acid	3,240.0	3,240.0	mg/kg
Oil acid	31,185.0	31,185.0	mg/kg
Linolec acid	73,845.0	73,845.0	mg/kg
Linolanic acid	3,645.0	3,645.0	mg/kg
Arachine	1,215.0	1,215.0	mg/kg

Chapter 7

No alterations of hippocampal neuronal number and synaptic bouton number in a transgenic mouse model expressing the β -cleaved C-terminal APP fragment

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ABSTRACT

Previous studies in the literature have resulted in conflicting reports on the potential neurotoxicity of the β -cleaved Alzheimer's disease C-terminal fragment (β -CTF) of β -amyloid precursor protein *in vivo*. To re-address this question by use of state-of-the-art stereological techniques, we analyzed transgenic mice expressing human β -CTF with the I45F mutation (SPA4CT) under control of the prion protein promoter. The transgene was expressed in hippocampus and cortex in large pyramidal neurons and in dentate gyrus granule cells. Proteolytic processing of β -CTF released A β . However, most of it remained uncleaved. Neurodegeneration was evaluated by investigating the numbers of hippocampal pyramidal and granule neurons, as well as the number of synaptophysin-immunopositive presynaptic boutons in the hippocampus of 15-month-old SPA4CT mice with design-based stereological techniques. The analyses showed that a fourfold higher expression of the transgene compared to murine APP levels had no effect on the numbers of both neurons and synaptophysin-immunopositive presynaptic boutons. These data implicate that expression of β -CTF per se is not neurotoxic, and that other mechanisms are responsible for the neurotoxic events in Alzheimer's disease brain.

INTRODUCTION

Alzheimer's disease (AD) is characterized by the presence of extracellular amyloid plaques, vascular amyloid, neurofibrillary tangles, and neurodegeneration. The amyloid deposits in AD brains consist mainly of the 40- to 42-residue β -amyloid (A β) peptides,^{1,2} which are derived in a two-step process by proteolytic cleavage from the larger β -amyloid precursor protein (APP).³ In the first step, β -secretase (BACE) cleavage releases the truncated ectodomain of APP and generates a ~12 kD C-terminal fragment (β -CTF or C-99).⁴ In the second step, β -CTF is processed by γ -secretase activity, generating A β (for review see ref. 5). For γ -secretase activity, presenilin- (PS) 1 and PS-2 are required which are multipass transmembrane proteins. Alternatively, cleavage within the A β domain of APP by α -secretase activity releases a secreted form of APP, prevents the formation of A β peptides and generates a ~10 kD C-terminal fragment (β -CTF or C-83) (see ref. 5 for review). Point mutations of the APP gene have been identified in some families with a rare autosomal dominant form of early onset AD.^{6,7} A further subset of familial AD cases is caused by mutations in the PS-1 gene on chromosome 14⁸ and the PS-2 gene on chromosome 1.^{9,10} The gain-of-function of PS mutations modifies APP processing and enhances the generation of A β 42.^{11,12}

The role of β -CTF in AD-related neurodegeneration has been controversially discussed in the literature. On one hand, it has been demonstrated *in vitro* that expression of full-length cDNA for human β -CTF is toxic in cultured neuronal and non-neuronal cell lines.¹³ Furthermore, it has recently been shown *in vitro* that β -CTF can induce astrocytosis, suggesting a role of β -CTF in promoting the production of proinflammatory mediators, nitric oxide, and subsequent reactive oxygen intermediates such as peroxynitrites by reactive astrocytes around senile plaques in AD.¹⁴ In addition, a recent *in vitro* study has suggested that the C-terminal β -secretase cleavage product of APP (i.e., β -CTF) – but not A β – mediates apoptosis of neurons caused by mutations

of the APP gene in early onset AD.¹⁵ On the other hand, expression of β -CTF in rat PC12 cells resulted in a significantly decreased vulnerability to H_2O_2 .¹⁶ Two studies *in vivo* have suggested age-dependent neuronal and synaptic degeneration – accompanied by impairments in learning and memory – in mice transgenic for the CTF of APP.^{17,18} In another study, however, neurodegeneration, gliosis, and reduced immunostaining for synaptophysin could only occasionally be observed in a large sample of mice transgenic for the 99-amino acid CTF of APP.¹⁹

The importance to understand the involvement of β -CTF in neurodegeneration and induction of astrocytosis is underlined by the fact that currently a major therapeutic strategy aims to decrease the production of A β in AD by inhibition of γ -secretase.^{4,20,21} With respect to this strategy it could recently be demonstrated that adult mice deficient in neuron-specific PS-1 (PS-1(n-/-)) had strongly decreased levels of endogenous brain amyloid peptides, concomitant with accumulation of endogenous β -CTF.²² Crossing PS-1(n-/-)-mice with mice transgenic for human mutant APP (APP[V717I]) resulted in double transgenic mice (APP[V717I]×PS1(n-/-)) not showing amyloid pathology and impairment of long-term potentiation as known for APP[V717I] mice. However, APP[V717I]×PS1(n-/-) mice demonstrated cognitive deficits similar to APP[V717I] mice, which were not seen in PS-1(n-/-) mice.²² The authors concluded that the use of γ -secretase inhibitors must be scrutinized further, considering potential neurotoxic effects of accumulating β -CTF (see also ref. 23–24).

On this basis we have carried out a re-evaluation of the question whether or not accumulation of β -CTF results in neurodegeneration (i.e., loss of synapses and/or loss of neurons) and gliosis *in vivo*. For doing so, we investigated – using state-of-the art stereological techniques – a transgenic mouse model expressing a construct with the APP signal peptide fused to the mutant I45F carboxyterminal 99 amino acids of APP under the control of the prion protein promoter.²⁵ A detailed description of the construct used can be found in Lichtenthaler et al.²⁶

MATERIALS AND METHODS

Animals

The transgene vector pBS/SPA4CT with a substitution of Ile-45 by phenylalanine was generated using the Quik Change Site Directed Mutagenesis Kit (Stratagene) as published previously.²⁷ This artificial mutation generated abundant processing of the C-terminal domain of APP and was therefore used to generate transgenic mice. The SPA4CT construct²⁸ contains the signal peptide (SP) of APP followed by leucine and glutamic²⁸ acid and the C-terminal 99 amino acids of APP (A4CT). Expression is under the control of the prion protein (pPrPhg) promoter. A NotI/SalI fragment containing the promoter and mutated cDNA sequence was isolated, purified and microinjected into the pronuclei of fertilized eggs (C57BL/6 x DBA) according to standard methods. Founders were backcrossed to C57BL/6 mice. Control animals were littermates of the transgenics. Over the entire period of investigation, no differences in health, activity pattern and maintaining of a normal dietary schedule were observed between transgenic and control animals. All experiments were performed according to the German animal protection law.

Western Blot Analysis

Transgenic (n=30) and control mice (n=40) of ages between 3 and 20 months were sacrificed by cervical translocation, and the brains removed from the skulls. One hemisphere per animal was immersion-fixed by 4% buffered formalin at 4° C and paraffin-embedded for immunohistochemical analysis (described below), and the other hemisphere was snap-frozen for biochemical analysis.

Snap frozen brain halves were homogenized in lysis buffer (120 mM NaCl, 50 mM Tris, 1% Triton X-100) including complete protease inhibitor cocktail (Boehringer Mannheim, Germany). Cellular debris was removed by centrifugation at 14,000 rpm at 4° C for 25 min. The proteins were run on 10% or 16% Tris-tricine SD-polyacrylamide gels (Novex, San Diego; CA) and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Western blots were probed with monoclonal antibody W0-229 and polyclonal anti APP-CT antibody.³⁰ For A β detection the blotted membrane was heated in boiling phosphate buffered saline (PBS; 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for 5 min to enhance the signal and blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 for 1 h. Afterwards, the primary antibody was added and incubated overnight at 4° C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Amersham) followed by ECL detection system (Amersham) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed on 4 μ m thick paraffin sections according to standard protocols. In brief, sections were deparaffinized in xylene and rehydrated. Treatment with 1% H₂O₂ in methanol was applied to block endogenous peroxidase activity. Microwave heating treatment in 0.01 M citrate buffer (pH 6.0) and pretreatment with 88% formic acid was performed in some sections and enhanced immunoreactivity. Sections were treated with fetal calf serum prior to the addition of the primary antibodies to block non-specific binding sites. This was followed by an overnight incubation with the primary antibodies at room temperature (RT). Staining was visualized using the ABC method, with a vectastain kit (Vector Laboratories, Burlingame, USA) and 0.3% diaminobenzidine (DAB) as chromogen. Counterstaining was carried out with hematoxylin. Immunohistochemistry was performed using the antibodies G2-10 (A β 40, 1:500), G2-4 (A β 40 and A β 42), W0-2 (A β 40 and A β 42), 27576 (APP-CT, 1:500) and anti-GFAP (1:400, DAKO) as described before.^{31,32}

Stereology

Five male, 15-month-old transgenic mice and five male age-matched controls were used to investigate the total numbers of hippocampal pyramidal and granule cells, the volumes of the hippocampal pyramidal and granule cell layer, the volume of the hippocampal white matter and the density of synaptophysin-immunopositive presynaptic boutons. The body weight of the transgenic mice was 45.6 $\pm$ 1.21 g (mean $\pm$ SEM), and was 40.4 $\pm$ 3.59 g for the control animals (unpaired t test: p = 0.207). Mice were anesthetized with chloral hydrate (10% aqueous solu-

Figure 1. Representative low-power photomicrographs of 100 μ m thick sagittal sections of the hippocampus of 15-month-old control mice (A) or SPA4CT transgenic mice (B). Sections were stained with cresyl violet and represent the same parasagittal level in the mouse brain (scale bar = 200 μ m). The insets show high-power photomicrographs of the pyramidal cell layers (area CA3) and the granule cell layers (dentate gyrus; DG) representative of the magnification at which the stereological estimates were made (scale bar = 15 μ m). The small squares indicate the positions at which the inset photomicrographs were taken. The dotted lines indicate the boundaries of the hippocampal pyramidal cell layer (CA1 to CA3).

Table 1. Details of the stereological analyses to estimate total numbers of neurons. *Obj.*, objective used; *B* and *H*, base and height of the optical disectors; *D*, distance between the optical disectors in mutually orthogonal directions *x* and *y*; *t*, measured actual average section thickness after histological processing; ΣOD , average sum of optical disectors used; ΣQ , average number of counted neurons; $CE_{pred}[n]$, average predicted coefficient of error of the estimated total numbers of neurons using the prediction method described by Schmitz (1998) and Schmitz and Hof (2000).

Type of neuron	Pyramidal cells (CA1–CA3)	Granule cells (dentate gyrus)
Obj	100×	100×
B (μm^2)	306.00	536.00
H (μm^2)	8	10
D (μm)	150	75
t (μm)	39.6	42.4
ΣOD	420	758
ΣQ	345	2577
$CE_{pred}[n]$	0.055	0.020

tion, 0.005 ml/g body weight, i.p.) and were sacrificed by intracardial perfusion fixation with formalin solutions as previously described.^{33–35} After opening the skulls, the heads of the animals were fixed in formalin solution for 24 h at 4° C. Afterwards the brains were removed, halved in the midsagittal line, and further fixed in formalin solution for 10 days at 4° C. Both brain halves were then cryoprotected in sucrose solution (10%, 20% and finally 30% sucrose in 0.1 M Tris–HCl buffer, 2×12 h per solution; 4° C) and were frozen in Tissue–Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands). Both hemispheres were stored at -70° C until further processing.

Left brain halves were used to investigate the total numbers of hippocampal pyramidal and granule cells, as well as the volumes of the hippocampal pyramidal and granule cell layer. These hemispheres were entirely cut on a cryostat (Type HM 500 OMV, Microm, Walldorf, Germany) to serial, 100 μm thick sagittal sections. Every third section was selected, mounted on gelatinized glass slides, dried and defatted with Triton X–100 (Merck, Darmstadt, Germany; 0.025%, 20 min) and stained with cresyl violet (0.01%, 20 min; cf. ref.^{34,35}). Slides were coverslipped using DePeX (Serva, Heidelberg, Germany).

Right brain halves were used to analyze the density of synaptophysin–immunopositive presynaptic boutons (SIPB). These brain halves were entirely cut on another cryostat (Leica CM 3050, Nussloch, Germany) into 30 μm thick coronal sections. Every eighth section was selected and used for synaptophysin immunohistochemistry as previously described.³⁶ Briefly, tri–buffered saline (TBS, 0.01 M) with 0.2% Triton X–100 (TBS–T) was used for washing and diluting antibodies. To minimize background staining, sections were pre–incubated in 5% normal goat serum (NGS; Sigma, Uithoorn, The Netherlands) at room temperature (RT) for 30 min. NGS (2.5%) was added to all solutions containing antibodies. A polyclonal antibody against synaptophysin (A0010, lot 019, Dako, Glostrup, Denmark) was used at a dilution 1:1000 overnight

Figure 2. Representative low-power photomicrographs of 30 μ m thick coronal sections of the hippocampus of 15-month-old control mice (A) or SPA4CT transgenic mice (B). Sections were processed for synaptophysin immunohistochemistry using a polyclonal antibody against synaptophysin (A0010, Dako, Denmark) and represent almost the same coronal level in the mouse brain (scale bar = 300 μ m). Below the low-power photomicrographs, three high-power photomicrographs each are shown of the granule cell layers (dentate gyrus; DG) and the pyramidal cell layers (areas CA1 and CA3), representative of the magnification at which the density of the synaptophysin-immunopositive presynaptic boutons was measured (scale bar = 15 μ m). The small squares indicate the positions at which the analyses were performed, as well as the high-power photomicrographs were taken. The dotted lines indicate the delineation of the hippocampal white matter.

at 4° C. After washing with TBS and TBS-T, the tissue was immersed in biotinylated goat anti-rabbit IgG (1:400; Jackson, West Grove, PA, USA) for 1.5 h at RT, followed by ABC-kit (1:800 for 90 min; Vectastain, Burlingame, CA, USA). After washing with TBS and TBS-T, the sections were incubated in 0.3% 3,3'-DAB (Sigma) containing 0.03% H₂O₂ in TBS-T for 10 min at RT. Sections were washed with TBS, mounted on gelatinized glass slides, dehydrated, cleared and coverslipped using Permount (Merck, Amsterdam, The Netherlands).

On sections of the left brain halves, the pyramidal and granule cell layers were delineated as depicted in Figure 1. No distinction was made between areas CA1 to CA3 (cf. ref 34). On sections of the right brain halves, the hippocampal white matter was delineated as depicted in Figure 2. Estimates of total numbers of neurons were carried out by means of the optical fractionator³⁷⁻³⁹ and a stereology workstation, consisting of a modified light microscope (type BX50; Olympus, Tokyo, Japan), Olympus UPlanApo objectives (10×, NA = 0.40; 100×, oil, NA = 1.35), a motorized specimen stage for automatic sampling (Maerzhaeuser, Wetzlar, Germany), an electronic microcator (Heidenhain, Traunreut, Germany), a CCD colour video camera (JAI, Glostrup, Denmark), a PC (IBM, Portsmouth, England) with framegrabber board (type Screen Machine II, Fast Multimedia, Munich, Germany) and stereology software (C.A.S.T.-Grid, Olympus; StereoInvestigator, MicroBrightField, Inc., Colchester, VT, USA) and a 17" television screen monitor (IBM). Using the sagittal sections of the left brain halves, all hippocampal pyramidal (CA1 to CA3) and granule cells were counted which nuclear top came into focus within optical disectors systematically-randomly spaced throughout the delineated brain regions. Counting was performed at a final magnification of ×3,600. In Table 1 all details of the counting procedure were summarized. Estimated total numbers of neurons were calculated from the number of counted neurons and the sampling probability.³⁷⁻³⁹

The same sections were used for investigating the volumes of the hippocampal pyramidal (CA1 to CA3) and granule cell layer. This was done by delineating and measuring the projection areas of these brain regions on all analyzed sections using the described stereology work station at a final magnification of ×360 (i.e., by using the 10× objective).

From the obtained data and the corresponding average actual section thickness after histological preparations (measured with the electronic microcator), total volumes were calculated by means of the Cavalieri's principle⁴⁰ considering overprojection.⁴¹ The hippocampal white matter volume was estimated on the synaptophysin-immunostained sections using the Cavalieri's principle as well. Since it was not possible to measure the average actual section thickness after histological preparations here, the original section thickness (30 µm) was considered for calculating the volumes.

The coronal sections of the right brain halves were used to analyze the density of the SIPB. Three regions within the hippocampus were analyzed, i.e., CA1, CA3 and the dentate gyrus (DG). Within the regions CA1 and DG, three randomly chosen areas of the systematically-randomly sampled sections were evaluated each, while in CA3 two randomly chosen areas were evaluated

as indicated in Figure 2. Five to seven sections per animal were analyzed, depending on the individual rostro-caudal extension of the hippocampus. The density of the SIPB was estimated as previously described.^{36,42} Briefly, an Olympus BH-2 microscope equipped with a 100 $\times$ oil immersion planachromatic objective and a 10 $\times$ projection lens was used. The microscope was equipped with a CCD video camera (Paes, Zoeterwoude, The Netherlands). Immunoreactive presynaptic boutons were detected by an image analysis system, slightly modified for detection of grayscale punctae (AnalySI-pro; Münster, Germany). All measurements were performed on a single focal plane. Shading error correction was performed before measurements to correct for irregularities in illumination of the microscopic fields. Three images per randomly chosen area were taken and stored. Background levels were equalized and the detection threshold was tested and kept at the same level for all samples. From these data, the mean SIPB density per μm^2 was calculated. The synaptophysin-immunoreactivity was restricted to small punctae with a morphological appearance that was not apparently different between animals or groups.

Statistical analysis

For each group, mean and standard error of the mean were calculated for all investigated variables (i.e., total numbers of hippocampal pyramidal and granule cells, volumes of the hippocampal pyramidal and granule cell layers, volumes of the hippocampal white matter, and densities of the hippocampal SIPB). Comparisons between the groups were performed with the Student's two-tailed *t* test. Statistical significance was established at $p < 0.05$. All calculations were performed using GraphPad Prism (Version 3.00 for Windows, GraphPad Software, San Diego, USA).

RESULTS

Western blot analysis

Total brain lysates of SPA4CT transgenic mice between 3 and 20 months, as well as age-matched control mice, were analysed by Western blot, either with monoclonal antibody W0-2 (A β 5-8) or polyclonal anti APP-CT antibody. As expected, in brain lysates of mice bearing the human transgene, a significant amount of human β -CTF was detected with both antibodies. The polyclonal anti APP-CT antibody recognized both the murine and the human APP C-terminus. The 12-kDa signal representing the human β -CTF was fourfold stronger than the endogenous murine β -CTF, as analyzed by densitometry using computer-assisted imaging software (Quantity One, Bio-Rad) (Figure 3A). There was no detectable difference in the expression level of β -CTF between transgenic and wildtype animals (Figure 3A). Levels of endogenous murine full-length APP, detected by Western blot with polyclonal anti APP-CT antibody, remained again unchanged in transgenic and control animals (Figure 3B). Analysis with the W0-2 antibody which detects human A β , revealed that the transgenic animals expressed the 12 kDa transgene, and additionally the 4 kDa A β band generated by γ -secretase cleavage. No age-related changes in the expression level of the β -CTF transgene or A β levels were found (data not shown). Neither the transgene nor A β were detected in brain lysates of non-transgenic control animals (Figure 3C).

Figure 3. Western blot analysis of SPA4CT transgenic mice and controls. *A*, Staining with a polyclonal anti APP-CT antibody, showing that transgenic β -CTF levels are higher than endogenous. *B*, Endogenous murine full-length APP stained with a polyclonal anti APP-CT antibody. *C*, W0-2 staining showing uncleaved C-terminal fragment of APP and A β . β -CTF, C-terminal fragment of APP; APPFL, full-length APP

Immunohistochemistry

Immunohistochemical analysis for human β -CTF using the polyclonal anti APP-CT serum in brain sections from transgenic mice revealed immunoreactivity in dentate gyrus granule cells, as well as in pyramidal neurons in hippocampus and cortical areas. Use of the polyclonal anti APP-CT serum resulted in a granular cytoplasmic immunostaining in the somatodendritic and axonal compartments (Figure 4). None of the transgenic mice showed any staining indicative of plaque-like structures or extracellular A β deposits. There was no indication for marked gliosis demonstrated by GFAP staining (not shown). Staining with the A β antibodies G2-10 (anti A β -40) and G2-4 (anti APP/A β) did not show any immunoreactivity. However, W0-2 elicited minor vesicular intraneuronal staining in some of the SPA4CT mice (not shown). The non-transgenic control mice were consistently negative for all antibodies.

Stereology

The mean total number of hippocampal pyramidal cells was $361,698 \pm 19,933$ (Mean $\pm$ SEM) for the transgenic mice, and $380,606 \pm 15,366$ for the wild type controls (i.e., a reduction by approximately 5% in the transgenic mice compared to the wild type controls).

Mean hippocampal granule cell number was $336,101 \pm 10,117$ for the transgenic mice, and $347,056 \pm 6,460$ for the controls (-3%). Transgenic mice exhibited hippocampi with mean volumes

of the pyramidal cell layer (CA1 to CA3), granule cell layer, and white matter of $0.332 \pm 0.026 \text{ mm}^3$ (controls: $0.352 \pm 0.015 \text{ mm}^3$; -5%), $0.187 \pm 0.014 \text{ mm}^3$ ($0.173 \pm 0.009 \text{ mm}^3$; +8%) and $2.591 \pm 0.076 \text{ mm}^3$ ($2.838 \pm 0.226 \text{ mm}^3$; -9%), respectively. Furthermore, the transgenic mice showed mean SIPB densities of $99.25 \pm 6.08/\mu\text{m}^2$ in CA1 (controls: $101.18 \pm 4.38/\mu\text{m}^2$; -2%), $102.02 \pm 5.87/\mu\text{m}^2$ in CA3 (109.21 ± 6.78 ; -6%), and $99.06 \pm 6.29/\mu\text{m}^2$ in DG ($100.71 \pm 3.79/\mu\text{m}^2$; -1.5%), respectively.

Thus, the transgenic mice showed a slight trend towards smaller hippocampi with less neurons and smaller SIPB density. However, these differences did not reach statistical significance. P-values were 0.474 for pyramidal cell number, 0.388 for granule cell number, and 0.527, 0.420, and 0.332 for the volumes of the pyramidal cell layer, the granule cell layer, and the white matter, respectively. Furthermore, p-values were 0.802, 0.448, and 0.827 for the SIPB densities in CA1, CA3, and DG, respectively. Since neither the volumes of the hippocampus nor the SIPB density showed any alteration, it is reasonable to interpret these data as indicating no alteration of the number (i.e., the product of density and volume) of SIPB in the hippocampus of the transgenic mice (cf ³⁶).

DISCUSSION

This is the first report using rigorous application of state-of-the-art stereological techniques to answer the question whether or not accumulation of β -CTF results in neurodegeneration (i.e., loss of synapses and/or loss of neurons) *in vivo*. In SPA4CT transgenic mice we observed expression of the C-terminal fragment of human APP in many large pyramidal neurons of the hippocampus and cortex, as well as in granule cells of the hippocampus. Western blot analysis of brain lysates of these animals revealed abundant C99 expression, representing human β -CTF with approximately fourfold higher relative levels of expression compared to the expression level of endogenous murine β -CTF in control animals. In addition, low

Figure 4. Immunohistochemical staining of brain sections of SPA4CT transgenic mouse brain using a polyclonal anti APP-CT antibody. A, Cortical section. B, Dentate gyrus granule cells. Counterstaining with hematoxylin. Bar = 50 μm (A) and 20 μm (B).

A β levels derived from the SPA4CT construct were found in all samples at all time points. No evidence for extracellular A β immunoreactivity or marked gliosis could be found using immunohistochemistry, even in 20 months old mice. Over the entire period of investigation, no differences in health, activity pattern and maintaining of a normal dietary schedule were observed between transgenic and control animals. Most importantly, we found only a slight trend towards smaller hippocampi with less neurons and smaller synaptophysin-immunopositive presynaptic bouton density in 15 months old transgenic animals compared to controls, with differences between 1.5% and 9% not reaching statistical significance. At the age of 15 months, no difference in body weight was found between transgenic and control animals (body weight not investigated at other time points). In general, our data further increase the understanding of the *in vivo* effects of β -CTF human APP, where previous studies in the literature have resulted in conflicting reports on the potential neurotoxicity of the β -CTF of APP *in vivo*. In their study, Sato and coworkers have shown that the major part of transgenic mice expressing β -CTF of human APP under the cytomegalovirus enhancer and chicken β -actin promoter lack signs for neurodegeneration.¹⁹ Data from two other studies, however, stand – at first glance – in contrast to our results. In a study investigating a transgenic mouse model¹⁷ in which the gene for the β -CTF of human APP was expressed under the control of the brain dystrophin promoter, Oster-Granite and coworkers found several darkly stained neurons both in the hippocampal granule and pyramidal cell layer of transgenic mice by inspection of toluidine blue stained semi-thin sections. On the electron microscopic level, the authors found axons containing increased numbers of neurofilaments relative to those of control mice, and membranous whorls in axonal and dendritic processes within the hippocampal pyramidal cell layer of transgenic mice numerous degenerating synapses and varying degrees of signs of degeneration in the hippocampus of all 25 transgenic mice studied at an age of more than 18 months, but only in 3 of 22 nontransgenic control littermates. The authors interpreted these data as support for the hypothesis that β -CTF of human APP is critically involved in the etiology of AD. In another study, the transgenic construct was identical to that described by Oster-Granite et al.¹⁷ except for the fusion of the Flag sequence (Asp-tyr-Ly-Asp-Asp-Asp-Asp-Lys), preceded by a methionine, to the N terminus of β -CTF18 which has shown to enhance the neurotoxicity of β -CTF *in vitro*⁴³ (see also¹⁷).

Homozygous Flag- β -CTF transgenic mice showed darkly stained neurons in the hippocampus already at an age of five months. Approximately 14 months old homozygous transgenic mice showed a reduction in the relative number of neurons with round, regularly-shaped white nuclei not staining darkly with the toluidine blue by approximately 35% compared to age-matched control animals.¹⁸ Interestingly, at the same time point, homozygous Flag- β -CTF transgenic mice were significantly impaired in cued, spatial and reversal performance of a Morris water maze task compared to age-matched controls.¹⁸ However, one should keep in mind that these finding might reflect the deleterious effects of Flag- β -CTF gene expression *in vivo*, which might not resemble the pathophysiological situation in AD.

The discrepancy between on the one hand the reports of Oster-Granite et al.¹⁷ and Berger-Sweeney et al.¹⁸ and on the other hand the data provided by Sato et al.¹⁹ and the present study might be due to several factors. Differences in the transgenic mouse models used – based, e.g.,

on the use of different promoters – might result in differences in the expression level of β -CTF and in different amounts of neurodegeneration among different transgenic mice whereas the different ages of the animals at the time point of investigation might also play a role. Furthermore, different research questions in the studies mentioned above, may have led to the use of different methods and techniques in the respective studies. In the present study, we focussed on the possible loss of neurons and/or synaptophysin-immunopositive presynaptic boutons whereas others have mainly focussed on the presence of degenerating neurons and synapses as a consequence of β -CTF expression. Additionally, some differences in the findings of the different studies could be based on different methods applied for analyzing the amount of neurodegeneration. In the present study, modern-design-based stereological techniques were used to quantify numbers of neurons and synaptophysin-immunopositive presynaptic boutons in different areas of the hippocampus of transgenic and nontransgenic mice. Application of these techniques in the present study resulted in a thorough investigation of the entire hippocampus, comprising inspection of several hundreds of microscopical fields per animal for counting neurons as well as of up to 168 microscopical fields per animal for analyzing SIPB density (for a comprehensive discussion regarding the quantification of synaptic numbers by investigating SIPB density and the volume of the corresponding brain region see.^{36,42}

To summarize, expression of β -CTF of human APP in transgenic mice resulted – if at all – only in minimal signs of neurodegeneration within the hippocampus when analyzing numbers of neurons and synaptophysin-immunopositive presynaptic boutons with modern-design-based stereological techniques, as well as signs of gliosis with immunohistochemistry. For obvious reasons, these data do not exclude the possibility that β -CTF of human APP might have neurotoxic effects in AD. Furthermore, these data do not exclude the possibility that the cognitive deficits found for the double transgenic mice (APP[V717I]×PS1(n/-)) investigated by Dewachter et al. (2002; see Introduction)²² are based on neurotoxic effects of accumulating β -CTF in this transgenic mouse model which might be investigated in the future in a similar manner as carried out here. In addition, one should keep in mind that the intracellular compartment of β -CTF generation in the model of Dewachter et al. (2002)²² and the present one might be different.

In conclusion, the results of this study contribute to clarify further which molecules generated in the amyloid cascade of AD are neurotoxic *in vivo*. As shown here, the C-99 fragment of APP was not able to induce a significant loss of synapses or neurons in the hippocampus of transgenic mice. Rather, recent research has raised the appealing hypothesis that the generation of high amounts of A β is crucial for neurodegeneration, which does not hold true for the SPA4CT model investigated here. Nevertheless, it is currently a matter of scientific debate whether intraneuronal or extracellular A β is neurotoxic, although it is clear that A β is generated intraneuronally. Intraneuronal A β accumulates prior to plaque induction in transgenic mice³¹ and in human post-mortem AD brain.⁴⁴ Furthermore, it has been shown that the A β domain is important for intraneuronal APP sorting.⁴⁵ Therefore, a pathological accumulation of intraneuronal A β could interfere with APP sorting and consequently with plaque formation.⁵ Indeed, Kienlen-Campard et al.⁴⁶ recently revealed that intraneuronal A β is neurotoxic in primary neuronal cell cultures. It is therefore to expect that further studies will confirm high amounts of intraneuronal A β as an important aspect of neurodegeneration in AD *in vivo*.

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Chapter 8

Hippocampal neuron loss exceeds amyloid plaque load in a transgenic mouse model of Alzheimer's disease

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ABSTRACT

According to the ‘amyloid hypothesis of Alzheimer’s disease’, β -amyloid is the primary driving force in Alzheimer’s disease pathogenesis. Despite the development of many transgenic mouse lines developing abundant β -amyloid-containing plaques in the brain, the actual link between amyloid plaques and neuron loss has not been clearly established, as reports on neuron loss in these models have remained controversial. We investigated transgenic mice expressing human mutant amyloid precursor protein APP751 (KM670/671NL and V717I) and human mutant presenilin-1 (PS-1 M146L). Stereologic and image analyses revealed substantial age-related neuron loss in the hippocampal pyramidal cell layer of APP/PS-1 double-transgenic mice. The loss of neurons was observed at sites of A β aggregation and surrounding astrocytes but, most importantly, was also clearly observed in areas of the parenchyma distant from plaques. These findings point to the potential involvement of more than one mechanism in hippocampal neuron loss in this APP/PS-1 double-transgenic mouse model of Alzheimer’s disease.

INTRODUCTION

The neuropathology of Alzheimer’s disease (AD) is characterized by aggregates of extracellular β -amyloid (A β), the formation of neurofibrillary tangles, neuronal and synaptic dysfunction, and loss of neurons and synapses.^{1,2} One of the most challenging aspects of the elucidation of AD pathogenesis is unraveling putative associations and causative links between these AD hallmarks. According to the ‘amyloid hypothesis of AD’, accumulation of A β in the brain is the primary driving force in AD pathogenesis.³ This hypothesis is supported by the fact that mutations in the amyloid precursor protein (APP) and in presenilins 1 (PS-1) and 2, causing early-onset cases of AD, modify APP processing and result in enhanced generation of A β .^{2,3} However, despite the development of various APP transgenic mouse models,^{4–6} the actual link between amyloid plaques and neuron loss has not been clearly established.^{7,8} In particular, reports on neuron loss in mice transgenic for APP or PS-1 have remained controversial.^{8–12} We have recently developed a novel transgenic mouse model of AD expressing human mutant APP751 (carrying the Swedish and London mutations KM670/671NL and V717I, Thy1 promoter) and human mutant presenilin-1 (PS-1 M146L, HMG promoter) in neurons.¹³ With the aim to rigorously investigate whether production and aggregation of A β or expression of mutant PS-1 may lead to neurotoxicity *in vivo*, we examined the hippocampus of APP/PS-1 double-transgenic mice as well as of mice transgenic only for human mutant PS-1 with detailed design-based quantitative and imaging techniques (the hippocampus was chosen due to its central role in the neuropathology of AD). In this article, we report a substantial age-related loss of hippocampal pyramidal cells in the APP/PS-1 double-transgenic mice. Neuronal loss within the hippocampus was greater than could be explained solely by the accumulation of extracellular β -amyloid. Rather areas at a distance from plaques clearly showed loss of neurons. Therefore, we propose that additional factors significantly contribute to neuron loss in this model of AD.

MATERIALS AND METHODS

Transgenic mice

The following groups of mice were examined: 4.5-months-old wild-type control mice ($n = 6$; body weight [BW] = 20.83 ± 0.70 g), 17-months-old wild-type control mice ($n = 6$; BW = 29.83 ± 2.36 g), 4.5-months-old transgenic mice expressing human mutant APP751 (carrying the Swedish and London mutations KM670/671NL and V717I, Thy1 promoter) and human mutant presenilin-1 (PS-1 M146L, HMG promoter; $n = 6$; BW = 19.67 ± 0.22 g), 17-months-old APP/PS-1 double-transgenic mice ($n = 7$; BW = 22.86 ± 0.80 g), and 17-months-old PS-1 single-transgenic mice ($n = 7$; BW = 30.43 ± 3.05 g). A detailed description of these transgenic mice has been given recently.^{13,14} APP/PS-1 double-transgenic mice were generated by crossing PS-1 (HMG PS1 M146L) homozygous mice to hemizygous APP (Thy1 APP751 SL) transgenic mice. The PS-1 mice had been back-crossed on a C57Bl6 background for more than six generations, whereas the APP mice were on a CBA (12.5%) $\times$ C57Bl6 (87.5%) background. Hemizygous PS-1 littermates were used in the present study. Control wildtype mice were on a C57Bl6 background. All mice were female and all experiments were performed in accordance with German animal protection law.

Tissue preparation, immunohistochemistry and lectin histochemistry

Mice were anesthetized with chloral hydrate and sacrificed by intracardial perfusion fixation with tyrode followed by the fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in phosphate buffer. Brains were removed rapidly, halved in the mediodorsal line, and post-fixed for 2 hours at 4 °C in the fixative, omitting the glutaraldehyde. Brain tissue was then cryo-protected by immersion in 30% sucrose in tris buffered saline at 4 °C overnight. Afterwards, brains were quickly frozen and stored at -80 °C until further processing. The right cerebral hemispheres were cut frontally into entire series of 30 μ m thick sections on a cryostat and were used for qualitative immunohistochemical visualization, and for the analysis of dying cells with the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick-end labeling) assay as recently described for frozen sections.¹⁵ The entire left hemispheres were cut sagittally and every tenth section was mounted on a glass slide, dried, defatted with Triton X-100, and stained with cresyl violet as described.¹⁶ Another series of every tenth section of each left hemisphere was used for immunohistochemical detection and quantification of extracellular A β and GFAP (i.e. glial fibrillary acidic protein as a marker for astrocytes). Immunohistochemistry was performed using standard immunofluorescence-labeling procedures. The following primary antibodies were used: monoclonal mouse anti-GFAP 1:1600 (Sigma, St. Louis, MO, USA), polyclonal rabbit anti-mouse GFAP 1:1600 (DAKO, Glostrup, Denmark), monoclonal mouse anti-neuronal nuclei antibody 1:100 (MAB377; Chemicon, Temecula, CA, USA), and rabbit anti-mouse polyclonal antiserum 730 1:5000 (against human A β and P3).¹⁷ Donkey anti-rabbit IgG Alexa Fluor 488 1:100, donkey anti-rabbit IgG Alexa fluor 594 1:100, and donkey anti-mouse IgG Alexa Fluor 488 1:100, (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. A third series of every tenth section of each left hemisphere was used for detection and quantification of extracellular A β by thioflavine S staining. Finally, lectin histochemistry with *Lycopersicon esculentum* (tomato lectin) and brightfield microscopy was used to identify microglia, according to standard protocols.¹⁸ Briefly, tissue

sections were blocked in methanol solution containing 2.5% of a 30% solution of hydrogen peroxide for 2 hours, incubated with lysis buffer (Hanks balanced saline solution containing 1% of each of the following: 1M MgCl₂, 1M CaCl₂, Tween 20, bovine serum albumin) for 2 hours, and left overnight in 1:100 biotinylated tomato lectin solution (Vector laboratories, UK) made up in lysis buffer. Following rigorous washes in TBS, lectin binding was visualized using the standard ABC-HRP method, with 3,3'-diaminobenzidine as chromogen. Negative control sections were incubated with tomato lectin solution containing the corresponding inhibitory substrate: 400mM N-acetylglucosamine.

Photography

All photomicrographs shown in Figure 1 were produced using a Nikon DXM 1200F digital camera (Nikon, Tokyo, Japan) and ACT-1 software (Nikon). Final images were constructed using Corel Photo-Paint v. 11. With respect to Figures 2, 3 and 5, photomicrographs were produced using an Olympus U-CMAD-2 digital camera. Between 6 and 8 images were captured for the composite in Figure 2 and 3, which represent the entire hippocampus. These images were made into one montage using image software (AnalySIS®-pro Münster, Germany). Images in Figure 5 were processed with Imaris imaging software (Bitplane, Zurich, Switzerland). Only minor adjustments of contrast and brightness were made, which in no case altered the appearance of the original materials.

Stereologic analyses

The granule and pyramidal cell layers were delineated on all cresyl violet-stained sections of the left hemisphere showing the hippocampus, as recently described.¹⁵ Estimates of layer volumes were made using Cavalieri's principle.¹⁹ Total numbers of neurons were evaluated with the Optical Fractionator.²⁰ Table 1 summarizes the details of the counting procedures. The series of sections from the left hemisphere of the 17-months-old APP/PS-1 double-transgenic mice stained for A β and GFAP by immunohistochemistry or with thioflavine S, were used to investigate the volume percentage of the hippocampal cell layers as well as of the entire hippocampus that was occupied by aggregated A β and surrounding astrocytes. This was performed using point counting methods as described in the literature.²¹ For technical reasons, one 17-months-old APP/PS-1 double-transgenic mouse could not be analyzed in this way. The percentage of the volume of the hippocampus occupied by aggregated A β and surrounding astrocytes in the 4.5-months-old APP/PS-1 double-transgenic mice was less than 1% and was therefore not considered further.

Statistical analysis

Differences between groups were tested with analysis of variance (ANOVA) followed by post-hoc Bonferroni's multiple comparison tests for pairwise comparisons. Correlations between numbers of neurons and plaque load were tested with linear regression analysis. Statistical significance was established at $P < 0.05$. All calculations were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Figure 1. Age-related astrocytosis, aggregation of A β aggregates, and loss of neurons in APP/PS-1 double-transgenic mice. Immunohistochemical analysis of the hippocampus of control mice (A and B), transgenic mice expressing human mutant presenilin-1 (PS-1 M146L, HMG promoter; C and D), and transgenic mice expressing human mutant amyloid precursor protein (APP) 751 (KM670/671NL and V717L, Thy1 promoter) and human mutant PS-1 (E to L). (A, C, E) Representative low-power photomicrographs from the hippocampus of 4.5-month-old mice, showing immunohistochemical staining for GFAP (red) and NeuN (green). (B, D, F) Representative low-power photomicrographs from the hippocampus of 17-month-old mice, showing immunohistochemical staining for GFAP (red) and NeuN (green). In all pictures, the dentate gyrus is seen on the left, and the CA3 and hilar region on the right. Note the strong increase in GFAP immunoreactivity in the APP/PS-1 double-transgenic mice. Furthermore, neuron loss was seen in the pyramidal cell layer of the 17-month-old APP/PS-1 double-transgenic mice (arrows in F). (G, H) Representative

low-power photomicrographs from the hippocampus of a 4.5-months-old (G) and a 17-months-old (H) APP/PS-1 double-transgenic mouse showing immunohistochemical staining for NeuN (red) and β -amyloid 40-42 (A β ; green). Note the age-related extracellular A β aggregation and neuron loss in the APP/PS-1 double-transgenic mice, particularly in the CA3 region. (I, K, L) Representative high-power photomicrographs from the CA2 region of 17-months-old APP/PS-1 double-transgenic mice, showing immunohistochemical staining for NeuN (red) and A β 40-42 (green) (I), GFAP (red) and A β 40-42 (green) (K), or GFAP (red) and NeuN (green) (L). There were regions free of neurons in the vicinity of the A β aggregations, but neuron loss was also found at distance from A β aggregations (arrows in I). The regions surrounding the A β aggregations were partly occupied by astrocytes (shown in K). However, the amount of neuron loss exceeded the accumulation of aggregated A β and surrounding astrocytes (arrows in L). Bar, 250 μ m in A to F, 150 μ m in G and H, 20 μ m in I to L.

RESULTS

Substantial Age-related Loss of Hippocampal Pyramidal Cells in APP/PS-1 Transgenic Mice

Our previous studies have shown that these APP/PS-1 double-transgenic mice express human mutant APP and PS-1 in various neuronal populations, including the hippocampal pyramidal and granule cells.¹³ Moreover, these mice produce intracellular A β and later develop extracellular A β aggregates, beginning in the cortex, subiculum and hippocampus at three months of age.^{13,14} In order to directly visualize the effects of expressing human mutant APP and PS-1 on A β aggregation and on the morphology of glial and neuronal cells in the mouse hippocampus, we performed several immunohistochemical analyses on frontal brain sections. Similar to that found in human AD, the amount of extracellular A β aggregates increase as the APP/PS-1 double-transgenic mice age (Figure 1, A–H). Chronic activation of microglia to form brain macrophages was evident in the aged APP/PS-1 animals, particularly related to plaques but also in the surrounding parenchyma (Figure 2). Importantly, we observed a clear age-related loss of neurons in the pyramidal cell layer of the hippocampus (Figure 1, F). We confirmed this qualitative finding quantitatively by conducting a detailed analysis with design-based stereology on cresyl violet stained sagittal sections of the other cerebral hemispheres (Figure 3 and 4). We found a substantial loss of pyramidal neurons in the hippocampus of 17-months-old APP/PS-1 double-transgenic mice compared to 4.5-months-old APP/PS-1 double-transgenic mice (-35%; $P < 0.001$) as well as compared to 4.5-months-old and 17-months-old wild-type controls (-34% [$P < 0.001$] and -25% [$P < 0.05$], respectively) and to 17-months-old PS-1 single-transgenic mice (-31%; $P < 0.001$). In contrast, there was no hippocampal neuron loss in 17-months-old PS-1 single-transgenic mice as compared to 17-months-old wild-type controls ($P > 0.05$), indicating that the expression of human mutant PS-1 did not result in neuron loss per se (Figure 4, D).

No Hippocampal Granule Cell Loss in APP/PS-1 Transgenic Mice

By applying the same methodology to the dentate gyrus, we did not observe hippocampal granule cell loss at 17 months of age in the APP/PS-1 double-transgenic mice as compared to 17-months-old PS-1 single-transgenic mice ($P > 0.05$), and compared to 17-months-old wild-type controls ($P > 0.05$) (Figure 4, C).

Less plaque load than the level of hippocampal pyramidal cell loss in APP/PS-1 Mice

We observed no increase in the local neuron density surrounding the A β aggregates (see Figure 1, E, F, H, I and L as well as Figure 3, C). This indicates that extracellular A β has not simply displaced neuronal cell bodies. Rather the observation of cells positive for both NeuN and TUNEL reactivity in the immediate vicinity of plaques (Figure 5) indicated that the observed hippocampal neuron loss in APP/PS-1 double-transgenic mice was – at least in part – connected to extracellular accumulation of A β . We therefore attempted to find a correlation between hippocampal neuron loss and extracellular accumulation of A β by quantifying the amount of extracellular A β aggregation within different layers of the hippocampus of the APP/PS-1 double-transgenic mice, using a combination of immunofluorescence imaging and stereologic analysis. Interestingly, we found no correlation between the numbers of hippocampal pyramidal neurons and the percentage of the volume of either the hippocampal pyramidal cell layer or the entire hippocampus occupied by aggregated A β and surrounding astrocytes (i.e., the plaque load) in the APP/PS-1 double-transgenic mice ($r^2 < 0.001$ and $r^2 = 0.080$, respectively; Figure 4, F and H). Importantly, we found the plaque load (averaging approximately 10%) to be considerably smaller than the level of hippocampal pyramidal cell loss in these mice (Figure 4, F; results obtained on thioflavine S stained sections were even somewhat smaller; details not shown). By comparison, a higher plaque load than presented here, was reported within the dentate gyrus of these APP/PS-1 double-transgenic mice in a previous study.¹⁴ The polymorph layer of the dentate gyrus was included for analysis in the study by Blanchard et al.,¹⁴ which showed a very high amount of plaque load (see also Figure 1, H). Our analysis focused on the granule cell layer of the dentate gyrus, showing a considerably smaller plaque load than the polymorph layer (Figure 1, H).

To further evaluate this difference between neuron loss and plaque load, we calculated ‘reconstructed’ numbers of hippocampal pyramidal cells of the 17-months-old APP/PS-1 double-

Figure 2. Representative high-power photomicrographs from the hippocampus of a 17-month-old APP/PS-1 double-transgenic mouse (A) and a 17-month-old control mouse (B), stained with tomato lectin histochemistry for chronically activated microglia. Note the strong activation of microglia in the hippocampus of the APP/PS-1 double-transgenic mouse related to plaques (white arrows) but also within the pyramidal cell layer (CA3; black arrows) and within the molecular layer (arrowheads). Microglial activation was not observed in the hippocampus of 4.5-month-old APP/PS-1 double-transgenic mice or in the hippocampus of the PS-1 single-transgenic mice (data not shown). Bar, 50 μ m

Table 1. Details of counting procedures used to evaluate total numbers of neurons. *Obj.*, objective used; *B* and *H*, base (μm^2) and height (μm^2) of the optical disectors; *D*, distance between the optical disectors in orthogonal directions *x* and *y* (μm); *t*, measured actual average section thickness after histologic processing (μm); ΣOD , average sum of optical disectors used; ΣQ , average number of neurons counted; $CE_{pred}[n]$, average predicted coefficient of error of the estimated total numbers of neurons.¹⁸

Type of neuron	Obj	B	H	D	t	ΣOD	ΣQ	$CE_{pred}[n]$
Pyramidal cells (CA1–CA3)	100×	591	5	100	8.4	342	1246	0.029
Granule cells (dentate gyrus)	100×	591	5	100	8.4	158	1617	0.025

transgenic mice on the assumption that the space within the hippocampal pyramidal cell layer occupied by aggregated A β and surrounding astrocytes would have contained neurons at the same mean neuronal density as the other parts of this layer. However, the mean ‘reconstructed’ number of hippocampal pyramidal cells of the APP/PS-1 double-transgenic mice was significantly smaller than the mean number of hippocampal pyramidal cells in the 4.5-months-old APP/PS-1 double-transgenic mice (-28%; $P < 0.01$) as well as in the 4.5-months-old wild-type controls (-26%; $P < 0.05$; Figure 4, K). This indicated a loss of neurons at sites of A β aggregates and surrounding astrocytes but also, most importantly, neuron loss distanced from A β aggregates. Accordingly, many regions within the hippocampal pyramidal cell layer of the 17-months-old APP/PS-1 double-transgenic mice were visibly free of neurons, even though they showed no signs of aggregated A β or astrocytes (Figure 1, L).

DISCUSSION

The expression of human mutant APP and human mutant PS-1 (but not human mutant PS-1 alone) caused substantial hippocampal pyramidal cell loss in the present transgenic model of AD. The lack of correlation between the numbers of pyramidal cells and the plaque load was in line with a study reporting little or no correlation between the level of neuron loss and the amount of extracellular A β in human AD.²² However, one cannot entirely exclude that the lack of correlation between the number of pyramidal cells and the plaque load within the hippocampus of the 17-months-old APP/PS-1 double-transgenic mice was due to interindividual variation in the number of hippocampal neurons between these mice before the onset of AD-like pathology (see supporting data on <http://www.amjpathol.org>). A previous study reported significant neuron loss in the hippocampal CA1 region in APP transgenic mice which seemed to correlate well with the amount of extracellular A β aggregates within the hippocampus.⁹ In that study,⁹ however, the correlation was based on inclusion of both homozygous and hemizygous transgenic mice in the calculations, with the latter showing hardly any extracellular A β aggregates and normal neuron numbers (see supporting data). Other studies have failed to find hippocampal neuron loss in APP and APP/PS-1 transgenic mouse models.^{10,11} Furthermore, a recent confocal analysis of APP transgenic mice and human *post mortem* tissue indicated that neuronal toxicity in AD is

primarily associated with thioflavine-S positive fibrillary A β deposits.⁸ However, correlations between the level of these deposits and actual neuron loss were not demonstrated. Altogether, we assume that – besides differences in quantitative analysis – the expression levels of APP and PS-1, the amount of generated A β , and the time interval between the beginning of A β formation and the analysis of neuron loss were the major factors responsible for the differences between the present and the previous studies^{8–11} on APP transgenic lines. Finally, it has been suggested that mutated PS-1 may already result in ‘degenerative-looking’ neurons in the hippocampus of transgenic mice.¹² The authors of this article concluded that PS-1 mutations may result in accelerated age-related neurodegeneration, although they did not conduct a detailed quantitative analysis of neuron loss. We could not confirm this hypothesis in our study.

Figure 3. Representative low-power photomicrographs of 30 μ m thick sagittal sections of the hippocampus of 17-month-old control mice (A), mice transgenic for human mutant presenilin-1 (B), and mice double-transgenic for human mutant APP751 and human mutant presenilin-1 (C), respectively. Sections were stained with cresyl violet and represent the same parasagittal level in the mouse brain (bar, 200 μ m). Insets, high-power photomicrographs of the granule cell layers (dentate gyrus; DG) and the pyramidal cell layers (area CA3) representative of the magnification at which the stereological estimates were made (bar, 15 μ m). Small rectangles depict the positions at which the inset photomicrographs were taken. Sections like these were used for quantification of hippocampal volumes and the total number of hippocampal pyramidal cells and granule cells.

Figure 4. Results of stereologic examinations. Control mice (C), transgenic mice expressing human mutant presenilin-1 (PS-1), and transgenic mice expressing human mutant amyloid precursor protein and human mutant PS-1 (APP/PS-1) were compared. (A, C, E, G, I) Results obtained for the left hippocampal granule cell layer (dentate gyrus). (B, D, F, H, K) Results obtained for the left hippocampal pyramidal cell layer (CA1-3). (A, B) Volumes of the cell layers. (C, D) Numbers of neurons. (E, F) Correlation between numbers of neurons (Y-axis) and the percentage of the volume of the corresponding cell layer occupied by aggregated A β and surrounding astrocytes in the APP/PS-1 double-transgenic mice (X-axis). (G, H) correlation between numbers of neurons (Y-axis) and the percentage of the volume of the entire hippocampus occupied by aggregated A β and surrounding astrocytes in the APP/PS-1 double-transgenic mice (X-axis). (I, K) 'Reconstructed' numbers of neurons, calculated assuming that in the hippocampus of the APP/PS-1 double-transgenic mice, the space within the corresponding cell layer occupied by aggregated A β and surrounding astrocytes would have contained neurons at the same mean neuronal density as the other parts of the corresponding cell layer. Dots represent individual data; lines represent mean data. M4.5, 4.5-months-old animals; M17, 17-months-old animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. In E to H, the curves indicate the upper and lower boundaries of the 95% confidence intervals of the corresponding regression lines. In all cases, the slope of the regression line was not statistically significantly different from zero ($P > 0.05$). Differences between groups were tested with analysis of variance (ANOVA), followed by post-hoc Bonferroni's multiple comparison tests for pairwise comparisons. Statistical significance was established at $P < 0.05$.

Figure 5. A cell (arrow) immunopositive for both NeuN (green; A) and TUNEL (red; B; merged in C) in the immediate vicinity of an A β aggregate (asterisk) in a 17-month old APP/PS-1 double-transgenic mouse, suggesting that neuronal death could be directly associated with accumulation of A β . Both pictures were taken at identical focal depth. Bar, 25 μ m

The results of the present study point to the involvement of more than one mechanism in hippocampal neuron loss in this APP/PS-1 double-transgenic mouse model of AD. The first mechanism (replacement of neurons by extracellular A β aggregates and surrounding astrocytes; Figure 1, I, K, and L) confirms the recently introduced concept of focal toxic A β deposits with a toxic gradient.⁸ In this respect, the role of reactive astrocytes around neuropil A β deposits is still enigmatic. On the one hand, the presence of astrocytes has been demonstrated to enhance A β -induced neurotoxicity in hippocampal cell cultures.²³ On the other hand, adult mouse astrocytes have been shown to degrade A β *in vitro* and *in vivo*.²⁴ Similarly, whereas chronically activated microglia are known to associate with A β plaques in human AD^{25,26} and in experimental conditions,²⁷ their presence additionally within the neuropil points to a more widespread and plaque-unrelated response in the APP/PS-1 double-transgenic mice. With respect to the neuron loss which cannot be explained by the concept of focal toxic A β deposits with a toxic gradient, it is tempting to speculate that the additional mechanisms are similar (or even identical) to those involved in alterations of synaptic, physiological and behavioral functions in other transgenic AD models before the onset of extracellular A β aggregation.^{28,29} In this context, abundant intraneuronal A β 40 and A β 42 with concurrent neuronal stress markers has been demonstrated prior to plaque formation in the present APP/PS-1 mouse model.^{13,14} Therefore, part of the hippocampal neuron loss may be caused by high levels of intraneuronal A β independent of extracellular A β aggregates. The soluble pool (intracellular and extracellular) of A β may also contribute to neurodegeneration, since soluble A β levels correlate better than insoluble (extracellularly aggregated) A β levels with the severity of AD³⁰ and since a spatial segregation of A β -oligomers and thioflavine-S positive deposits has recently been described.³¹

Thus, APP/PS-1 mice allow us to determine the exact roles of intracellular and extracellular A β in hippocampal neuronal toxicity and to investigate the mechanisms of neuron loss *in vivo*.

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Chapter 9

Age-related loss of synaptophysin immunoreactive presynaptic boutons within the hippocampus of APP751^{SL}, PS1^{M146L} and APP751^{SL}/PS1^{M146L} transgenic mice

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ABSTRACT

Neuron and synapse loss are important features of the neuropathology of Alzheimer's disease (AD). Recently we observed substantial age-related hippocampal neuron loss in APP751^{SL}/PS1^{M146L} transgenic mice but not in PS1^{M146L} mice. Here we investigated APP751^{SL} mice, PS1^{M146L} mice and APP751^{SL}/PS1^{M146L} mice for age-related alterations in synaptic integrity within hippocampal stratum moleculare of the dentate gyrus (SM), stratum lucidum of area CA3 (SL) and stratum radiatum of area CA1–2 (SR) by analyzing densities and numbers of synaptophysin-immunoreactive presynaptic boutons (SIPB). Wild-type mice, APP751^{SL} mice and PS1^{M146L} mice showed similar amounts of age-related SIPB loss within SM, and no SIPB loss within SL. Both APP751^{SL} mice and PS1^{M146L} mice showed age-related SIPB loss within SR. Importantly, APP751^{SL}/PS1^{M146L} mice displayed the severest age-related SIPB loss within SM, SL and SR, even in regions free of extracellular A β deposits. Together these mouse models offer a unique framework to study the impact of several molecular and cellular events caused by mutant APP and/or mutant PS1 on age-related alterations in synaptic integrity. The observation of age-related SIPB loss within SR of PS1^{M146L} mice supports a role of mutant PS1 in neurodegeneration apart from its contribution to alterations in A β generation.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder comprising cognitive and memory deterioration, progressive impairment of activities of daily living, and several neuropsychiatric symptoms.¹ The neuropathology of AD is characterized by disturbances in neural circuits, such as loss of neurons and synapses, and by protein aggregations of β -amyloid and hyperphosphorylated tau.^{2–4} Accumulating evidence has indicated a crucial role for failure and loss of synapses in AD pathophysiology.^{2,4,5–7} Postmortem morphologic studies on AD neuropathology have demonstrated significantly reduced synaptic connectivity in brain regions as the neocortex and hippocampus.^{3,6,7} Reductions in synaptic densities showed a strong correlation with cognitive decline in AD.⁵ However, the reasons for the reduced connectivity in AD remain poorly understood.⁸ According to the ' β -amyloid (A β) hypothesis of AD', A β accumulation is the primary driving force in AD pathogenesis. This hypothesis is supported by the fact that mutations in the amyloid precursor protein (APP) and in presenilins 1 (PS1) and 2, causing early-onset cases of AD, modify APP processing and result in enhanced generation of A β .⁴ We have developed a transgenic mouse model of AD expressing human mutant APP751 (carrying the Swedish and London mutations KM670/671NL and V717I, Thy1 promoter; APP751^{SL} mice), human mutant presenilin-1 (PS1 M146L, HMG promoter; PS1^{M146L} mice) or both human mutant APP751 and human mutant presenilin-1 (APP751^{SL}/PS1^{M146L} mice) in neurons^{9–12} (see also^{8,13}). Similar to what happens in human AD, the amount of extracellular A β deposits as well as the level of reactive astrogliosis increase as these APP751^{SL} mice and APP751^{SL}/PS1^{M146L} mice age.^{9–12} Furthermore, the APP751^{SL}/PS1^{M146L} mice show substantial age-related loss of hippocampal pyramidal cells.¹² Importantly, the amount of neuron loss exceeded the amount

of A β deposits and associated reactive astrogliosis in these mice, indicating that the observed neuron loss was at least in part independent of A β deposits.¹² Given the popular belief that extracellular A β deposits are the cause of neuronal degeneration in AD,⁴ these findings have prompted modification of the original "amyloid cascade" hypothesis for AD.^{13,14}

It was the aim of the present study to test the hypothesis that these APP751^{SL} mice, PS1^{M146L} mice and APP751^{SL}/PS1^{M146L} mice show region-specific, age-related alterations in synaptic integrity within the hippocampus, with region-specific differences among the investigated genotypes. Another aim was to determine to what extent these alterations in synaptic integrity were due to A β deposits within the hippocampus and to hippocampal neuron loss. The investigations were carried out by two-photon laser scanning microscopy, computerized image analysis and design-based stereology, focusing on densities and numbers of synaptophysin-immunoreactive presynaptic boutons (SIPB). Analysis of SIPB is a well established approach for investigating alterations in synaptic integrity *in vivo* both in our^{15,16} and in many other laboratories.^{17–21}

MATERIALS AND METHODS

Animals

The following groups of either 4.5-month-old (M4.5) or 17-month-old (M17) female mice were examined in the present study: wild-type control mice (C57Bl6 background; M4.5: n=6; M17: n=6), APP751^{SL} mice (transgenic mice expressing human mutant APP751 carrying the Swedish and London mutations KM670/671NL and V717I; Thy1 promoter; C57Bl6 [87.5%] $\times$ CBA [12.5%] background; M4.5: n=6; M17: n=3), PS1^{M146L} mice (transgenic mice expressing human mutant presenilin-1 [PS1 M146L]; HMG promoter; back-crossed on a C57Bl6 background for more than six generations; M4.5: n=6; M17: n=6) and APP751^{SL}/PS1^{M146L} mice (APP/PS1 double-transgenic mice carrying the aforementioned APP and PS1 mutations; M4.5: n=6; M17: n=6). All mice were generated at the SanofiAventis Centre de Recherche de Paris (Vitry sur Seine, France) (for details see^{9–12}). The APP751^{SL}/PS1^{M146L} mice were generated by crossing homozygous PS1 mice to hemizygous APP751^{SL} mice. In the present study hemizygous PS1^{M146L} littermates were used. All experiments were performed in accordance with the German animal protection law.

Tissue processing

Mice were anesthetized with chloral hydrate and sacrificed by intracardial perfusion fixation with tyrode followed by fixative containing 4% paraformaldehyde, 15% picric acid, and 0.05% glutaraldehyde in phosphate buffer (see¹² for details). Brains were removed rapidly, halved in the midsagittal line, and post-fixed for 2 hours at 4° C in the fixative, omitting the glutaraldehyde. Brain tissue was then cryoprotected by immersion in 30% sucrose in Tri-buffered saline at 4° C overnight. Afterward, brains were quickly frozen and stored at -80° C until further processing. The right hemispheres were cut into entire series of 30 μ m thick frontal sections on a cryostat (Leica CM 3050; Leica, Nussloch, Germany). Afterwards these series were divided

into 10 subseries of every 10th section each. The hippocampus was found on five to eight sections per subseries depending on the individual rostrocaudal extension of the hippocampus. Four subseries of sections per animal (from the wild-type control mice, the PS1^{M146L} mice and the APP751^{SL}/PS1^{M146L} mice but not from the APP751^{SL} mice) were used in a former study¹² to investigate age-related alterations in neuron numbers, the amount of A β deposits, apoptotic cell death and microglia activation within the hippocampus. The left hemispheres were not used for the present study.

Investigation of alterations in SIPB morphology due to A β deposits

To investigate normal SIPB morphology and its alterations due to A β deposits, one subseries of sections per animal was used for immunohistochemical detection of both synaptophysin and β -amyloid (monoclonal mouse anti-synaptophysin antibody, 1:2,000; Chemicon, Temecula, CA; rabbit anti-730 antibody detecting β -amyloid and P6,²² 1:5,000; generous gift from Dr. G. Multhaup). Briefly, fluorescent immunohistochemistry was carried out in a free floating manner using standard protocols. Sections were incubated overnight at 4° C after rinsing with tri-buffered saline (TBS) and TBS with Triton-X 100 (TBS-T) and blocking of unspecific staining with 10% fetal calf serum and 4% non-fat-dry-milk in TBS. On the following day the sections were rinsed and incubated with secondary antibodies for 1.5 hours at room temperature. Donkey anti-mouse IgG Alexa Fluor 488 antibody (1:100; Molecular Probes, Eugene, OR, USA) and donkey anti-rabbit IgG Alexa Fluor 594 antibody (1:100; Molecular Probes), were used as secondary antibodies. Sections were mounted on gelatinized glass slides, dried and coverslipped using 80% glycerol in PBS. Sections were analyzed with standard fluorescence microscopy and two-photon laser scanning microscopy (TPLSM). TPLSM was performed as previously described²³ using a microscope objective (60 $\times$, water dipping, numerical aperture [NA] 1.0) connected to an upright Nikon E600FN microscope (Nikon Corporation, Tokyo, Japan). If needed, further magnification was achieved by an optical zoom in the scan head. To remove noise each image was filtered applying the Kalman filtering procedure on three subsequent images during experiments. The fluorescent secondary antibody Alexa 488 was mainly visible in the green channel, whereas Alexa 594 was only visible in the red channel and the obtained images (coded green and red, respectively) were combined into single images if warranted.

Analysis of SIPB densities

To analyze SIPB densities in various hippocampal subregions, one subseries of sections per animal was used for immunohistochemical detection of synaptophysin (monoclonal mouse anti-synaptophysin antibody; 1:2,000; Chemicon; avidin-biotin immunoperoxidase labeling) (Figure 1A to D). The M.O.M. immunodetection kit (Vector Laboratories, Peterborough, UK) was used according to the manufacturer's protocol to minimize background labeling and labeling of intraparenchymal IgG molecules. Control experiments comprised incubations without primary antibody and incubations without use of the M.O.M. immunodetection kit (Vector). All incubations were performed in a free-floating manner under exactly identical conditions.

After labeling procedures, sections were mounted on gelatinized glass slides, dehydrated, coverslipped and coded. The code was not broken until all analyses were completed.

SIPB densities were analyzed as previously described^{15,16} within three subregions of the hippocampus, stratum moleculare of the dentate gyrus (SM), stratum lucidum of area CA3 (SL) and stratum radiatum of area CA1–2 (SR) (Figure 1E). Three randomly chosen areas per section were evaluated within SM and SR each, and two randomly chosen areas within SL (Figure 1E). Three high-power photomicrographs were taken from each evaluated area at approximately 2 μm below the upper surface of the sections (i.e., when the SIPB were clearly in focus) with a digital camera (F-view, Olympus, Tokyo, Japan) attached to an Olympus AX-70 microscope (100 $\times$ oil objective, Olympus UplanApo, NA = 1.35). This procedure resulted in 45 to 72 photomicrographs for SM and SR each per animal (3 photomicrographs $\times$ 3 areas $\times$ [5 to 8] sections), and in 30 to 48 photomicrographs for SL per animal (2 photomicrographs $\times$ 3 areas $\times$ [5 to 8] sections) (Figure 1F to H). In case of the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice photomicrographs were exclusively taken at regions free of A β deposits and A β aggregation-related disturbances in normal SIPB morphology. A β deposits were clearly recognizable as round/oval structures not immunoreactive for synaptophysin and surrounded by multiple enlarged SIPB and disturbed neuropil (Figure 1I). SIPB were detected by image analysis using AnalySI-pro software (Soft Imaging System, Münster, Germany), slightly modified for detection of grayscale punctae as previously described.¹⁶ All settings were kept identical for all analyses. Shading error correction was performed before measurements were done to correct for irregularities in illumination of the microscopic fields. Furthermore, background levels were equalized. From these data, mean particle densities per μm^2 were calculated. To enable the elimination of noise signal and to differentiate between possible artifacts and specific SIPB, the normal size of SIPB was evaluated separately for SM, SL and SR in a pilot experiment. For doing so, the area occupied by individual particles with a morphological appearance of SIPB was measured separately within SM, SL and SR in three wild type mice using the AnalySI-pro software (Soft Imaging System). A total of 400 particles were measured per hippocampal subregion. SIPB areas were on average $0.33 \pm 0.12 \mu\text{m}^2$ (mean $\pm$ S.D.) in SM, $1.02 \pm 0.38 \mu\text{m}^2$ in SL and $0.36 \pm 0.13 \mu\text{m}^2$ in SR. The different SIPB size can be seen in Figure 1F to H. A region-specific range of [mean $\pm$ (2 $\times$ S.D.)] was considered as normal SIPB size, and these settings were kept identical for all analyses. Size measurements of all SIPB investigated in the present study indicated no differences in average SIPB size between the various groups of mice. Additional pilot studies (not described here) indicated that average SIPB size was independent from the plane of section (i.e., identical results were obtained on frontal, sagittal and horizontal sections of mouse brains).

Measuring volumes of SM, SL and SR

Volumes of SM, SL and SR were evaluated on the sections immunohistochemically processed for synaptophysin detection with avidin–biotin immunoperoxidase labeling with the aid of another subseries of sections per animal stained with cresyl–violet (Nissl) staining (0.01%) as previously described.^{12,16} Briefly, the Nissl-stained sections were laid onto the corresponding adjacent sections processed with immunohistochemistry and were exactly aligned. Then SM,

SL and SR were delineated according to the literature²⁴ (Figure 1K). Delineation was carried out with a stereology workstation and StereoInvestigator software (MicroBrightField, Williston, VT, USA) as previously described.¹⁶ For doing so, the Nissl-stained sections were brought into focus. After delineation these sections were removed, and the sections processed with immunohistochemistry were brought into focus and the boundaries of SM, SL and SR were verified. Volumes of SM, SL and SR were calculated with Cavalieri's principle^{25,26} from the projection area measurements and the average actual section thickness after histologic processing. The latter was measured with the electronic microcator of the stereology software and a 40× oil objective (Olympus UplanApo; NA = 1.00) as described.²⁶

Calculation of SIPB numbers

Hippocampal subregion-specific SIPB numbers were calculated by multiplying the individual SIPB density data with the corresponding volume data. Note that these SIPB numbers were not unbiased since SIPB densities were analyzed in one focal plane and thus in two dimensions (2D) instead of in three dimensions (3D).²⁶ Thus, the calculations did not take the dimensions of the investigated parameters (i.e., $1/\mu\text{m}^2$ [SIPB density] and mm^3 [volume]) into account. Importantly, this did not affect the results of the present study since for all groups of mice, similar distribution plots were obtained for individual SIPB size (not shown) and the average SIPB size was independent from the plane of section. Calculating unbiased total numbers of SIPB was not the aim of the present study.

Calculation of corrected SIPB numbers

For the analysis of the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice the calculation of SIPB numbers resulted in theoretical numbers assuming that A β deposits did not influence normal SIPB morphology (since SIPB densities were exclusively measured at regions free of A β deposits and A β aggregation-related disturbances in normal SIPB morphology as explained above). To take the impact of A β deposits on SIPB numbers into account, the percent volumes of SM, SL and SR occupied by A β deposits and A β aggregation-related disturbances in normal SIPB morphology (as depicted in Figure 1I) were analyzed with point counting methods^{26,27} on the sections immunohistochemically processed for synaptophysin detection with avidin-biotin immunoperoxidase labeling. Point counting was performed on the stereology workstation with the 40× oil objective and a distance of 50 μm between the points in mutually orthogonal directions X and Y for SM and SR (and 20 μm for SL, respectively). Corrected SIPB numbers for the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice were calculated by subtracting the volume occupied by A β deposits and A β aggregation-related disturbances in normal SIPB morphology from the total volume of the investigated hippocampal subregions, and by multiplying this value with the mean SIPB density found within the corresponding hippocampal subregion. Thus, corrected SIPB numbers for the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice were smaller than the corresponding uncorrected SIPB numbers. For the wild-type control mice and the PS1^{M146L} mice the corresponding uncorrected SIPB numbers could serve as corrected SIPB numbers as well. This was due to the fact that these mice did not develop A β deposits.

Figure 1. Representative photomicrographs of frontal sections through the hippocampus of a 17-month-old wild-type control mouse (A, E to H, K), a 17-month-old APP751^{SL} mouse (B), a 17-month-old PS1^{M146L} mouse and a 17-month-old APP751^{SL}/PS1^{M146L} mouse (D, I), showing immunoreactivity for synaptophysin (A to K). Aβ deposits within the hippocampus of the APP751^{SL} mouse and the APP751^{SL}/PS1^{M146L} mouse are indicated by arrows in B and D, respectively. The small squares in E represent the areas where the high-power photomicrographs for quantitative synaptophysin-immunoreactive presynaptic bouton (SIPB) analysis were taken within stratum moleculare of the dentate gyrus (SM), stratum lucidum of area CA3 (SL) and stratum radiatum of area CA1–2 (SR). Corresponding high-power photomicrographs depict larger SIPB within SL (G) than within SM (F) and SR (H). Aβ deposits were clearly recognizable as round/oval structures not immunoreactive for synaptophysin and surrounded by multiple enlarged SIPB and disturbed neuropil (I). The dotted line in I indicates the delineation of Aβ deposits as performed for the analysis of SIPB densities in regions free of Aβ deposits and Aβ aggregation-related disturbances in normal SIPB morphology. In K the projection areas of SM, SL and SR are shown. Scale bar = 250 μm in A to E and K, 20 μm in F to H, and 10 μm in I.

Statistical analysis

For each group of mice mean and standard error of the mean were calculated for all investigated variables (hippocampal subregion-specific SIPB density, volume of the subregion, SIPB number and corrected SIPB number if applicable). Comparisons between the groups were performed separately for SM, SL and SR with two-way analysis of variance (two-way ANOVA; four genotypes and two age groups per genotype) followed by Bonferroni post-tests to compare replicate means focusing on age-related effects. Statistical significance was established at $P < 0.05$. All calculations were performed using GraphPad Prism (Version 4.00 for Windows, GraphPad Software, San Diego, USA).

Photography

Pictures shown in Figure 1A to E and 1K were generated with the stereology workstation using the Virtual Slice tool of the StereoInvestigator software which automatically created high-resolution seamless image montages composed of approximately 100 individual field-of-view visualized at high resolution. Photomicrographs shown in Figure 1F to I were taken with the Olympus AX-70 microscope. Pictures shown in Figure 2 and 3 were acquired using TPLSM. Image stacks were reconstructed into three-dimensional images followed by colocalization analysis with Imaris software (Bitplane, Zurich, Switzerland). Final figures were constructed using Corel Draw Version 11.0 (Corel, Ottawa, Canada) and Adobe Photoshop Version 7.0 (Adobe, San Jose, CA, USA). Only minor adjustments of contrast and brightness were made, which in no case altered the appearance of the original materials.

RESULTS

Alterations in SIPB morphology due to A β deposits

A β deposits within the hippocampus were detected in APP751^{SL} mice at M17 and in APP751^{SL}/PS1^{M146L} mice at M4.5 and M17. No synaptophysin immunoreactivity was observed within the core of the A β deposits. Regions directly adjacent and surrounding the A β deposits showed substantial alterations in synaptophysin immunoreactivity. Specifically, considerably enlarged SIPB were detected (up to 8 μ m in diameter) close to the A β deposits (Figure 2). Three-dimensional reconstruction of TPLSM image stacks showed that some of these enlarged SIPB were surrounded by A β (Figure 2).

SIPB densities

Two-way ANOVA showed statistically significant age- and genotype-related differences in mean SIPB densities within SM, SL and SR (all p values of two-way ANOVA are summarized in Table 1). Bonferroni post-tests showed a significant age-related decline in mean SIPB densities within SM for the PS1^{M146L} mice (on average -19%) and the APP751^{SL}/PS1^{M146L} mice (-33%), within SL only for the APP751^{SL}/PS1^{M146L} mice (-27%), and within SR for the PS1^{M146L} mice (-24%) and the APP751^{SL}/PS1^{M146L} mice (-31%) (data and p values of Bonferroni post-tests shown in Figure 3A to C).

Volumes of SM, SL and SR

Two-way ANOVA indicated significant age-related differences in mean volumes of SM, SL and SR (see Table 1 for all *p* values). In case of SM and SR differences in mean volumes were also significant among the investigated genotypes. However, Bonferroni post-tests showed a significant age-related decline only in the mean volume of SM in the APP751^{SL}/PS1^{M146L} mice (on average -26%) (Figure 3D to F).

SIPB numbers

As for mean SIPB densities and the mean volumes of SM, SL and SR two-way ANOVA indicated significant age-related differences in mean SIPB numbers within all investigated hippocampal subregions (see Table 1 for all *p* values). In case of SM and SR differences in mean SIPB numbers were also significant among the investigated genotypes. Bonferroni post-tests showed a significant age-related decline in mean SIPB numbers within SM for the wild-type control mice (on average -19%), the PS1^{M146L} mice (-24%) and the APP751^{SL}/PS1^{M146L} mice (-49%), within SL only for the APP751^{SL}/PS1^{M146L} mice (-33%), and within SR for the PS1^{M146L} mice (-30%) and the APP751^{SL}/PS1^{M146L} mice (-38%) (for data and *p* values of Bonferroni post-tests see Figure 3G to I).

Corrected SIPB numbers

Two-way ANOVA revealed statistically significant age- and genotype-related differences in mean corrected SIPB numbers within SM, SL and SR (the *p* values of two-way ANOVA are given in Table 1). Bonferroni post-tests showed a significant age-related decline in mean corrected SIPB numbers within SM for all investigated genotypes (wild-type control mice: -19% on average; APP751^{SL} mice: -19%; PS1^{M146L} mice: -24%; APP751^{SL}/PS1^{M146L} mice: -56%), within SL only for the APP751^{SL}/PS1^{M146L} mice (-42%) and within SR for all investigated genotypes except for the wild-type control mice (APP751^{SL} mice: -32%; PS1^{M146L} mice: -30%; APP751^{SL}/PS1^{M146L} mice: -51%) (data and *p* values of Bonferroni post-tests shown in Figure 3K to M).

Table 1. Results of two-way ANOVA (*p* values).

		SM	SL	SR
SIPB density	Age	< 0.001	0.009	0.001
	Genotype	< 0.001	0.014	0.001
	Interaction	0.001	0.004	0.015
Volume of the subregion	Age	0.001	0.002	< 0.001
	Genotype	< 0.001	0.290	0.036
	Interaction	0.114	0.755	0.440
SIPB number	Age	< 0.001	0.004	< 0.001
	Genotype	< 0.001	0.077	< 0.001
	Interaction	0.018	0.059	0.024
Corrected SIPB number	Age	< 0.001	0.001	< 0.001
	Genotype	< 0.001	0.016	< 0.001
	Interaction	0.004	0.056	0.002

SM, hippocampal stratum moleculare of the dentate gyrus; SL, hippocampal stratum lucidum of area CA3; SR, hippocampal stratum radiatum of area CA1-2

Figure 2. Representative high-power photomicrographs obtained by two photon laser scanning microscopy (TPLSM), showing alterations in synaptic integrity caused by A β deposits within hippocampal subregion SR of a 17-month-old APP751^{SL}/PS1^{M146L} mouse. A β immunoreactivity is shown in red in A, D and G, synaptophysin immunoreactivity at the corresponding locations and focal planes in green in B, E and H, and merged pictures in C, F and I. Note the disturbed synaptophysin-immunoreactive presynaptic bouton (SIPB) morphology within and in the near vicinity of the A β deposits (arrows). The SIPB-free regions around the A β deposits (arrowheads in E and F) are most probably caused by astrocytes surrounding the A β deposits. Interestingly, several enlarged SIPB were entirely surrounded by β -amyloid (arrows in I). In K to M a three dimensional reconstruction of a representative image stack acquired with TPLSM is shown, displaying synaptophysin (green) and A β (red) immunoreactivity within SR of a 17-month-old APP751^{SL}/PS1^{M146L} mouse. The dotted lines in K represent the position within the X-Y view at which the Y-Z view (L) and the X-Z view (M) were generated. The depicted enlarged synaptophysin-immunoreactive presynaptic bouton is surrounded by a rim of A β immunoreactivity. Scale bar = 100 μ m in A to C, 50 μ m in D to I, and 5 μ m in K to M.

DISCUSSION

Alterations in SIPB morphology due to A β deposits

In the present study enlarged SIPB were found in the near vicinity of A β deposits within the hippocampus of the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice. This finding is in line with several studies in the literature reporting the presence of enlarged SIPB in AD patients²⁸ as well as in APP transgenic and APP/PS1 transgenic mice.^{21,29–33} Furthermore, dystrophic neurites with swollen varicosities in the near vicinity of A β deposits as well as spine loss and shaft atrophy have been reported in APP transgenic and APP/PS1 transgenic mice.^{29,30,34,35} Of particular interest was the observation of a close spatial relationship between aggregated A β and enlarged SIPB in the present study. One may speculate that A β deposits are involved in the formation of enlarged SIPB. In this respect, a study by Phinney et al.³⁶ indicated that A β deposits in APP23 mice may attract dystrophic axonal terminals. However, it is also possible that enlarged SIPB are involved in the production or secretion of A β . Recent evidence indicating that axonally

Figure 3. Results of the quantitative investigations in the hippocampus. Analysis of hippocampal stratum moleculare of the dentate gyrus (SM; A, D, G, K), stratum lucidum of area CA3 (SL; B, E, H, L) and stratum radiatum of area CA1–2 (SR; C, F, I, M) of wild-type control mice (WT), APP751^{SL} mice (APP), PS1^{M146L} mice (PS1) and APP751^{SL}/PS1^{M146L} mice (APP/PS1) for age-related alterations in synaptophysin-immunoreactive presynaptic bouton (SIPB) densities within regions free of A β deposits and A β aggregation-related disturbances in normal SIPB morphology (A, B, C), volumes of SM, SL and SR (D, E, F), SIPB numbers within regions free of A β deposits and A β aggregation-related disturbances in normal SIPB morphology (G, H, I) and corrected SIPB numbers considering the amount of A β deposits within SM, SL and SR (K, L, M). Results of 4.5-month-old mice (M4.5) are represented by open bars; those of 17-month-old mice (M17) by closed bars. Data are given as mean and S.E.M.. Comparisons between the groups were performed separately for SM, SL and SR with two-way analysis of variance (two-way ANOVA; *p* values summarized in Table 1) followed by Bonferroni post-tests to compare replicate means focusing on age-related effects (*p* values provided as * [*p* < 0.05], ** [*p* < 0.01] or *** [*p* < 0.001] in A, B, C, D, G, H, I, K, L and M). Significant differences in mean data are indicated (M17 vs M4.5) above each graph. ROI, region of interest; Corr., corrected. SIPB numbers were calculated by multiplying the individual SIPB density data with the corresponding volume data not considering the dimensions (see Materials and methods for details).

transported APP can give rise to A β and that A β is subsequently released from presynaptic sites and deposited in A β deposits *in vivo*³⁷ may argue for the latter speculation. Furthermore, APP, presenilins and synaptophysin have been shown to co-localize at the growth cones of

developing cultured neurons.³⁸ In summary it is reasonable to conclude that A β deposits play an important role in degeneration of presynaptic structures and have therefore considerable impact on local synaptic connectivity.

Alterations in SIPB densities and SIPB numbers in APP751^{SL} and APP751^{SL}/PS1^{M146L} transgenic mice

The wild-type control mice investigated in the present study did not show significant differences in mean SIPB densities within hippocampal subregions SM, SL and SR and in mean volumes of these subregions between 4.5 months and 17 months of age. Furthermore, an age-related decline in mean SIPB number was only observed within SM but not within SL and SR of the wild-type control mice. These data are in line with previous reports in the literature.^{19,20,39,40}

Transgenic expression of human mutant APP and human mutant PS1 in the investigated mice had significant, region-specific effects on mean SIPB densities and SIPB numbers within the investigated hippocampal subregions. Compared to the age-matched wild-type control mice, the APP751^{SL} mice showed transgene-induced age-related loss of SIPB only within SR (results of the PS1^{M146L} mice are separately discussed below). Within SM a similar amount of age-related loss of SIPB was observed in the wild-type control mice, the APP751^{SL} mice and the PS1^{M146L} mice. Furthermore, none of these mice showed an age-related loss of SIPB within SL. In contrast, the APP751^{SL}/PS1^{M146L} mice displayed transgene-induced age-related loss of SIPB within SM, SL and SR. Besides this, there was no difference between the wild-type control mice and the APP751^{SL} mice with respect to age-related loss of SIPB within SR as long as

A β deposits were not taken into account. In contrast, the APP751^{SL}/PS1^{M146L} mice showed a transgene-induced age-related loss of SIPB even in regions within SM, SL and SR free of A β deposits (discussed separately below).

Based on the analysis of SIPB densities within the hippocampal subregion SM of APP_{wt} I63 and APP_{Ind} H6 transgenic mice, Mucke et al.²⁰ suggested evidence for A β -induced synaptotoxicity even in the absence of A β deposits in APP transgenic mice. This is in contrast to the findings of the present study in which age-related loss of SIPB in the absence of A β deposits was found only for the APP751^{SL}/PS1^{M146L} mice but not for the APP751^{SL} mice. Methodological differences might explain this discrepancy between the findings of Mucke et al.²⁰ and the present study. Mucke et al.²⁰ correlated their SIPB density data with the overall hippocampal plaque load and found no significant correlation between these parameters in their APP transgenic mice. Based on this lack of correlation these authors concluded that A β is synaptotoxic even in the absence of plaques in their APP transgenic mice.²⁰ However, in the present study no correlation between SIPB densities within SM and overall hippocampal plaque load was found in the APP751^{SL} mice as well as in the APP751^{SL}/PS1^{M146L} mice (not shown). Rather correlation analyses between plaque load and other neuropathologic findings in transgenic mouse models of AD may be misleading, as outlined recently.¹² Another reason for the discrepancy between the findings of

Mucke et al.²⁰ and the present study might be differences in the transgenic constructs, i.e. in the introduced mutations and/or the promoters used. In this regard it is important to note that the APP transgenic mice investigated by Mucke et al.²⁰ showed a significantly reduced SIPB density within SM compared to wild-type controls already at two to four months of age. This might reflect a specific neurodevelopmental defect in the APP transgenic mice investigated by these authors. In contrast, in the present study the APP751^{SL} mice had the highest mean SIPB densities as well as (except for SL at M4.5) the highest mean SIPB numbers within SM, SL and SR among the investigated genotypes at both M4.5 and M17. This might indicate synaptotrophic effects of APP in the APP751^{SL} mice investigated in the present study as known from other transgenic mice expressing different forms of human APP.^{41,42} In this regard it is important to note that Takeuchi et al.³³ found slightly higher SIPB densities within hippocampal subregions SM and SR of APP^{K670N/M671L} transgenic mice and of APP^{K670N/M671L}/PS1^{M146L} double transgenic mice than within SM and SR of both wild-type control mice and PS1^{M146L} transgenic mice at 12 months of age. These data are fully in line with the results of the present study at M4.5. Wong et al.⁴³ found, compared to wild-type controls, increased densities of vesicular acetylcholine transporter immunoreactive presynaptic boutons within SR of APP^{KM670/671NL} transgenic mice but neither within SR of PS1^{M146L} transgenic mice nor of APP^{K670N/M671L}/PS1^{M146L} double transgenic mice at 8 months of age. Except for the results for the APP^{K670N/M671L}/PS1^{M146L} double transgenic mice these data are in line with the findings of the present study at M4.5 as well. Takeuchi et al.³³ and Wong et al.⁴³ restricted their analyses to 12-months-old animals (or to 8-months-old animals, respectively). Thus, these studies cannot be compared to the present study with respect to age-related alterations in hippocampal SIPB densities based on transgenic expression of human mutant APP and/or human mutant PS1 in mice. The same holds true for a study by Dodart et al.⁴⁴ in which SIPB densities within several hippocampal subregions of 12-month-old APP^{V717F} transgenic mice were investigated (on average no difference between APP^{V717F} transgenic mice and wild-type control mice).

Furthermore it should be mentioned that King and Arendash²¹ observed a decline of approximately 40% in mean SIPB densities within hippocampal subregions SM and SR of wild-type control mice between 3 and 19 months of age, and an approximately 40% increase in SIPB densities within SM and SR of APP⁶⁹⁵ transgenic mice. Accordingly these authors found SIPB densities within SM and SR of 19-month-old APP⁶⁹⁵ transgenic mice approximately twice as high as within SM and SR of 19-month-old wild-type controls.²¹ These data are neither in line with the results of the present study nor with the findings of Mucke et al.²⁰ and other reports in the literature concerning age-related alterations in synaptophysin immunoreactivity within the hippocampus of wild-type rodents.^{19,20,39,40} Besides differences in the transgenic constructs the reason for this discrepancy might be due to different methodologies. Seemingly the image analysis approach of King and Arendash²¹ did not focus on the analysis of SIPB densities within regions free of A β deposits and A β aggregation-related disturbances in normal SIPB morphology. Furthermore, it cannot be excluded that the intense synaptophysin staining demonstrated by King and Arendash²¹ for APP⁶⁹⁵ transgenic mice at 19 months of age was affected by intraparenchymal IgG molecules (blocked by using the M.O.M. immunodetection kit [Vector] in the present study).

Finally Phinney et al.³⁶ observed that A β deposits in APP23 mice attracted dystrophic axonal terminals to ectopic locations which may lead to disruption of normal neuronal connectivity. Accordingly, the intense synaptophysin immunoreactivity at the periphery of neuritic plaques seen in the APP751^{SL} mice and the APP751^{SL}/PS1^{M146L} mice investigated in the present study as well as described in the literature (discussed above) might reflect neurotropic properties of A β deposits. One might even ask the question whether this mechanism could result in reduced SIPB numbers in areas that should have received the projections. The results obtained on the APP751^{SL} mice in the present study may answer this question. Provided that the main mechanism in SIPB loss in regions free of A β deposits would have been the result of inappropriate redirection of projections to cluster around the A β deposits (and consequently resulting in deficits in the areas that should have received the projections), the APP751^{SL} mice should have shown significant differences in SIPB densities and numbers (as compared to the wild-type control mice) in regions free of A β deposits. However, as shown in Figure 3 this was not the case.

In summary the results of the present study are in line with several reports in the literature, and differences to other reports can be explained. Importantly, to the best of our knowledge, the results of the present study are the first ones demonstrating both quantitative and qualitative differences between the simultaneous transgenic expression of human mutant APP and human mutant PS1 and the single transgenic expression of either human mutant APP or human mutant PS1 in mice on age-related loss of SIPB within the hippocampus. Potential reasons for these differences are discussed in the next paragraphs.

Alterations in SIPB densities and SIPB numbers in PS1^{M146L} transgenic mice

Of particular importance was the finding of a transgene-induced age-related reduction in both mean SIPB density and mean SIPB number within the hippocampal subregion SR (CA1–2) of the PS1^{M146L} mice in the present study. To the best of our knowledge these are the first results demonstrating a region-specific effect of transgenic expression of human mutant PS1 on age-related loss of SIPB within the mouse hippocampus.

Presenilins are considered to be the catalytic subunits of the gamma-secretase complex, but they are also essential for the fine tuning of the immunological system and for memory and synaptic plasticity.⁴⁵ AD-associated presenilin mutations clearly affect gamma-secretase cleavage and thereby enhance A β generation.¹⁰ According to previous observations by Duff et al.,⁴⁶ expression of mutant PS1 itself may generate murine A β . We have previously investigated the presence of human A β in the PS1^{M146L} mice and found no A β signal in ELISA blots using a monoclonal antibody (WO-2) against human A β .¹⁰ However, we cannot exclude that there was at least some generation of endogenous murine A β (possibly not detectable by the antibody used in our previous study) in the PS1^{M146L} mice. On the other hand, we detected considerable amounts of A β in the APP751^{SL} mice.¹⁰ The fact that we found more age-related SIPB loss within SM and SR of the PS1^{M146L} mice than within SM and SR of the APP751^{SL} mice would argue that other factors rather than A β were the driving force in SIPB loss in the PS1^{M146L} mice. In this respect, it is important to address that presenilins affect intracellular calcium stores,

protein trafficking and protein turnover and neuronal signaling.⁴⁷ Consequently, it has been proposed that destabilization of calcium signaling has a role in neurodegeneration in AD.⁴⁷ Mutations in PS1 and PS2 are known to perturb endoplasmic reticulum calcium homeostasis such that greater amounts of calcium are released upon stimulation.^{48,49} Furthermore, Herms et al.⁵⁰ showed deregulation of intracellular calcium homeostasis by decreased capacitative calcium entry (CCE) in hippocampal neurons dissociated from newborn mice transgenic for mutant PS1^{A246E} (CCE being a refill mechanism allowing entry of extracellular calcium ions through plasma membrane channels that are tightly regulated by and even physically linked to intracellular stores⁵¹). Importantly, calcium-induced formation of reactive oxidative species (ROS) and mitochondrial swelling were found higher in isolated rat mitochondria from the CA1 than from the CA3 region.⁵² Furthermore, synaptosomal proteins from PS1M146V knock-in mice displayed increased oxidative stress compared with synaptosomal proteins from wild type mice.⁵³ Begley et al.⁵⁴ observed evidence of disturbed calcium homeostasis and mitochondrial dysfunction in synaptosomes prepared from mice transgenic for mutant PS1; it is reasonable to hypothesize that similar to the knock-in mice studied by LaFontaine et al.⁵³, synaptosomal proteins were also affected by increased oxidative stress in the transgenic mice investigated by Begley et al.⁵⁴. Moreover, it was recently shown that expression of the PS1 mutations M146V, I143T, and deletion of exon 9 (D9) in human neuronal cells *in vitro* significantly increased the activation of glycogen synthase kinase 3- β (GSK3- β).⁵⁵ *In vivo*, GSK3- β phosphorylates kinesin light chains (KLC) and causes the release of kinesin-I from membrane-bound organelles (MBO), leading to a reduction in kinesin-I-mediated fast axonal transport.⁵⁶ Consistent with such a deficit in kinesin-I-mediated fast axonal transport, densities of synaptophysin-containing vesicles and mitochondria were found reduced in neuritic processes of hippocampal neurons expressing mutant PS1^{M146V} *in vitro*.⁵⁵ On the basis of these findings, it is attractive to speculate that the observed transgene-induced age-related loss of SIPB within SR of the PS1^{M146L} mice in the present study was connected to transgene-induced alterations in cellular calcium homeostasis, fast axonal transport and the formation of ROS. At least the latter mechanism might occur preferentially within the hippocampal CA1 region, therefore triggering age-related SIPB loss preferentially within SR. Interestingly, perturbations in calcium homeostasis and alterations in GSK3- β activation caused by mutations in PS1 might be connected to each other. Calcium signaling activates the phosphatase calcineurin and induces movement of NFATc proteins into the nucleus, whereas nuclear import of NFATc proteins is opposed by kinases such as GSK3- β .⁵⁷

Besides these mechanisms, recent studies indicated that presenilins also mediate cleavage of synaptic-associated adhesion molecules such as nectin1 α ⁵⁸ and cadherins.⁵⁹ However, it remains to be determined whether PS1 cadherin/catenin interaction or PS-mediated cleavage of adhesion molecules play a functional role in synapse formation or modulate synaptic activity.⁶⁰ Thus, one can only speculate whether altered PS1-mediated cleavage of synaptic-associated adhesion proteins influenced synaptic function and integrity and was involved in the reported age-related loss of SIPB within SR of the PS1^{M146L} mice.

Finally it should be mentioned that in a recent study on 3-month-old mice in which the M146V mutation of PS1 was knocked in into the endogenous mouse PS1 gene, Wang et al.⁶¹ found

impaired hippocampus-dependent contextual learning and reduced adult neurogenesis in the dentate gyrus. The latter result is in line with earlier findings of increased cell cycle arrest caused by PS1 mutations overexpressed in cells *in vitro*.⁶² On the other hand, total numbers of hippocampal granule cells were found unchanged compared to wild-type controls in mice transgenic for the same PS1 mutation as investigated by Wang et al.⁶¹ as well as in mice in which the human PS1 mutations M233T and L235P were knocked in into the mouse PS1 gene.^{12,63} Thus, the possible impact of reduced neurogenesis in the dentate gyrus of PS1^{M146V} knock-in mice on hippocampus-dependent contextual learning remains elusive.

In summary the finding of a transgene-induced age-related loss of SIPB within the hippocampal subregion SR of the PS1^{M146L} mice in the present study can be explained by potential mechanisms of mutant PS1 reported in the literature. One can regard the findings of the present study as among the first *in vivo* evidence for a role of mutant PS1 in neurodegeneration without concomitant neuron loss, apart from its contribution to alterations in A β generation. This might serve as the basis for further modifying the amyloid hypothesis of neurodegeneration in AD.¹⁴

Alterations in SIPB densities and SIPB numbers in APP751^{SL}/PS1^{M146L} transgenic mice independent of A β deposits

As explained above, there was no difference between the wild-type control mice and the APP751^{SL} mice with respect to age-related loss of SIPB as long as A β deposits were not taken into account. It can therefore not be excluded that local development of A β deposits fully accounted for the loss of SIPB seen within hippocampal subregion SR of the APP751^{SL} mice at M17. In contrast, the APP751^{SL}/PS1^{M146L} mice showed a transgene-induced age-related loss of SIPB even in regions within SM, SL and SR free of A β deposits. This phenomenon can be due to several interacting mechanisms: (i) The aforementioned potential actions of mutant PS1 (at least within SR). (ii) Local neuron loss. Our previous study on the same APP751^{SL}/PS1^{M146L} mice showed a substantial age-related loss of hippocampal pyramidal cells (on average approximately 30%).¹² Loss of hippocampal pyramidal cells resulted most probably in a decreased number of dendrites in SL and SR. Thus, one cannot exclude that the combined action of hippocampal pyramidal cell loss and A β deposits within SL and SR fully accounted for the loss of SIPB seen within these regions of the APP751^{SL}/PS1^{M146L} mice. At M17 these mice showed a transgene-induced age-related reduction in mean SIPB numbers of 33% in regions within SL (and of 38% in regions within SR, respectively) free of A β deposits, and an approximately 30% loss of pyramidal cells within the hippocampus.¹² (iii) Neuron loss in regions with heavy projections to the areas analyzed. In this regard, we have previously shown that age-related neuron loss occurred within the hippocampal pyramidal cell layer but not within the hippocampal granule cell layer of the APP751^{SL}/PS1^{M146L} mice,¹² i.e. exactly the same mice as investigated in the present study. The mossy fibers (i.e., the axons of the hippocampal granule cells) project to the hippocampal pyramidal cells (mainly to CA3). Thus, the effects seen in the stratum lucidum of area CA3 (SL) of the APP751^{SL}/PS1^{M146L} mice cannot be explained by neuron loss in the region projecting to SL. Furthermore, age-related loss of SIPB was found in SR of the PS1^{M146L} mice, which did not show age-related neuron loss in any hippocampal area.¹² Preliminary evidence from microscopic inspection suggested age-related neuron loss

in the entorhinal cortex of the APP^{751SL}/PS1^{M146L} mice but not in the PS1^{M146L} mice (not shown) which showed SIPB loss within SM. Together these data indicate that loss of neurons at regions with heavy projections towards the areas analyzed in the present study was of limited impact on age-related SIPB loss. (iv) Action of soluble A β . Soluble A β species have been demonstrated to induce neurotoxicity which is likely to provoke neuropathology reminiscent of AD,^{64,65} see also ^{2,66–68}. In this regard, a 12% reduction in total dendritic length of hippocampal granule cells was found in 3-month-old APP^{V717F} transgenic mice, i.e., prior to amyloid deposition, in a recent study by Wu et al.⁶⁹ Certain subsets of granule cells (superficially located granule cells in the dorsal blade of the dentate gyrus) even exhibited a 23% loss of total dendritic length at 3 months of age.⁶⁹ The authors of this study hypothesized that these alterations were due to elevated levels of soluble forms of A β , induced by the overexpression of APP.⁶⁹ Comparably, one may speculate that at least part of the 30% (49% minus 19% age-related loss of SIPB numbers seen within SM of the wild-type control mice), transgene-induced age-related reduction in SIPB numbers in regions free of A β deposits within SM of APP^{751SL}/PS1^{M146L} mice is due to elevated levels of soluble A β . (v) Intraneuronal A β . Previous reports have shown that intraneuronal A β preceded the formation of A β deposits in APP^{751SL}/PS1^{M146L} mice⁹ and that elevated levels of intraneuronal A β were associated with several alterations in normal cell homeostasis.^{11,14,64,70} Moreover, intraneuronal A β has been reported to substantially affect synaptic function²⁶ and integrity.⁷¹ (vi) A chronic inflammation status. Previously, we reported widespread microglia activation within the hippocampal neuropil of APP^{751SL}/PS1^{M146L} mice which was only partly related to A β deposits.¹² Thus, one could argue that microglia activation might be an early pathogenic event preceding loss of SIPB and possibly having a causative role in SIPB loss.^{72,73}

With respect to the potential impact of both soluble forms of A β and intraneuronal A β on SIPB loss in hippocampal regions free of A β deposits, analysis of SIPB densities and numbers in the hippocampus of APP^{SL}/PS1KI mice⁷⁰ will be important. This is due to the fact that mutations in PS1 have been shown to enhance gamma-secretase activity towards elevated A β ₄₂ generation.^{74–76} The A β ₄₂/A β ratio was approximately 0.2 in the APP^{751SL} mice and approximately 0.4 in the APP^{751SL}/PS1^{M146L} mice investigated here,¹¹ and was increased to approximately 0.85 in APP^{SL}/PS1KI mice which showed profound hippocampal neuron loss and intraneuronal accumulation of A β already at 10 months of age.⁷⁰ We hypothesize that in the hippocampus of APP^{SL}/PS1KI mice profound SIPB loss will also be found in regions free of A β deposits, and the comparison of results from APP^{751SL}/PS1^{M146L} mice and APP^{SL}/PS1KI mice will provide novel insights into the impact of transgenic expression and “knock-in” expression of mutations of PS1 on impairments of synaptic integrity in AD.

Relevance of the findings of the present study to human AD

Loss of hippocampal synaptophysin immunoreactivity was established as an early marker in human AD but did not show spatial correlation with A β deposits.⁷⁷ By simultaneously investigating the formation of neurofibrillary tangles and the relative amount of synaptophysin mRNA within the same neurons a number of studies indicated evidence for a tangle-dependent

mechanism of hippocampal synaptophysin loss in AD.^{78–80} However, the formation of tangles accounts for only a small proportion of loss of hippocampal neurons in AD, at least within area CA1.⁸¹ Accordingly, the formation of tangles is most probably not the predominant mechanism of hippocampal synaptophysin loss in AD. This is supported by the finding of no local correlation between the degree of synaptophysin-like immunoreactivity and CD-1 immunoreactive neurons within the hippocampus of brains from patients with AD (with the CD-1 antibody recognizing tangles, dystrophic neurites in and around A β deposits and neuropil threads).⁷⁷ Rather Callahan et al.⁸⁰ recently found that hippocampal CA1 neurons free of both tangles and immunohistochemical detectable tau phosphorylation at Ser 262, 356 and 396/404 in brains from patients with AD (Braak stage V–VI⁸²) showed a statistically significant reduction of more than 20% in the relative amount of synaptophysin mRNA compared with corresponding neurons in brains from controls (Braak stage I–II).

These data indicate that hippocampal synaptophysin loss in AD seems to be multifactorial. In this regard the simultaneous analysis of the APP751^{SL} mice, the PS1^{M146L} mice and the APP751^{SL}/PS1^{M146L} mice investigated in the present study offers a unique framework to test hypotheses on the impact of several molecular and cellular events caused by transgenic expression of human mutant APP and/or human mutant PS1 on age-related alterations in synaptic integrity. Firstly, the transgene-induced age-related SIPB loss seen within hippocampal subregion SR of the APP751^{SL} mice might be almost completely due to A β deposits. This hypothesis can be tested by vaccination or other approaches focusing on the prevention of A β aggregation. Secondly, the transgene-induced age-related SIPB loss seen within SL and SR of the APP751^{SL}/PS1^{M146L} mice might be almost completely due to the combined action of A β deposits and neuron loss. This offers for the first time the possibility to address the question whether alterations in synaptic integrity precede neuron loss in a transgenic animal model of AD. Thirdly, the transgene-induced age-related SIPB loss seen within SR of the PS1^{M146L} mice cannot be attributed to A β deposits or to neuron loss. This offers for the first time the possibility to study actions of mutant PS1 in neurodegeneration apart from its contribution to altered APP processing and A β generation. Finally, the transgene-induced age-related SIPB loss seen within SM of the APP751^{SL}/PS1^{M146L} mice can also neither be attributed to A β deposits nor to neuron loss. As age-related SIPB loss within SM of the PS1^{M146L} mice did not exceed age-related SIPB loss within this hippocampal subregion of the wild-type control mice, one might speculate that the age-related SIPB loss within SM of the APP751^{SL}/PS1^{M146L} mice which could not be explained by A β deposits and neuron loss was not due to actions of mutant PS1. Thus SIPB loss in APP751^{SL}/PS1^{M146L} is most probably due to complex interactions between the A β deposits, neuronal loss locally or at distance, generation of intraneuronal and soluble A β and other PS1 mediated actions.

In summary the transgenic mouse models as investigated in our study can be seen among the best animal models of AD and provide for the first time the possibility to investigate hippocampal subregion-specific differences in interactions between neuron loss, A β deposits, soluble A β , intraneuronal A β , and PS1 mediated actions on alterations in synaptic integrity. Future experiments are likely to have further significant impact on the field of AD research.

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Chapter 10

General discussion

Mechanisms of Neuronal Loss in Aging and Alzheimer's Disease

Discussion

To prevent or treat neuronal alterations and neuronal loss in aging and Alzheimer's disease (AD), it is crucial to identify the underlying molecular and cellular mechanisms. Although our knowledge about these mechanisms is still rather limited, the findings described in this thesis have provided novel insights. Testing the hypotheses described in Chapter 1 led to answers to several research questions and elucidated issues in the field of aging and AD:

First, I have shown that quantitative neuromorphologic analyses on the brain should be carried out on sets of sections systematically and randomly sampled, spanning the entire brain region of interest. This resulted into new standards for quantitative neuromorphological investigations.^{1,2}

Second, I have shown that these types of neuron in the mouse brain with age-related decline in the rate of nDNA repair showed accumulation of unrepaired nDNA damage during aging without age-related loss of neurons. In contrast, those types of neuron without an age-related decline in the rate of nDNA repair showed no accumulation of unrepaired nDNA damage during aging but an age-related loss of neurons. These findings indicate important cell-type-specific differences during brain aging and suggest that attenuation of age-related accumulation of nDNA damage in neurons may be reached through several strategies.

Third, I found that long term caloric restriction of mice reduced the relative amount of nDNA damage in hippocampal neurons during aging and reduced age-related shrinkage of hippocampal neurons. Transgenic upregulation of an endogenous antioxidant however did not reduce these age-related alterations in mice.

Fourth, I showed by using novel transgenic mouse models of AD that (a) transgenic expression of the C99 terminal fragment of human APP did not result in age-related loss of hippocampal neurons or synaptophysin-immunoreactive presynaptic boutons (SIPB) within the hippocampus, (b) transgenic expression of human mutant APP did not result in age-related loss of SIPB in the hippocampus, (c) transgenic expression of human mutant PS1 resulted in age-related loss of SIPB in hippocampal area CA 1–2 but not in age-related loss of hippocampal neurons, (d) transgenic expression of both human mutant APP and human mutant PS1 resulted in age-related formation of extracellular A β aggregations, which were associated with age-related hippocampal neuron loss and with disturbances of synaptic integrity, and (e) mechanisms other than extracellular A β aggregations significantly contributed to age-related hippocampal neuron loss in mice transgenic for both human mutant APP and human mutant PS1. Together these findings resulted in modifications of the original A β cascade hypothesis of AD pathogenesis.^{1,3}

Fifth, my findings on mouse models of aging and AD indicated, in parallel to findings in humans,⁴ that AD is accompanied by dramatic neuron loss and destruction of certain neuronal circuitries while normal aging is not. I observed that aging influenced synaptic structure in selective brain regions and was connected with accumulation of nDNA damage in selective types of neuron.

FUTURE DIRECTIONS

Nuclear DNA damage and synaptic alterations in aging

Aging affects subcellular processes in types of neuron in the hippocampus and cerebral cortex rather than inducing loss of neurons. The observed inverse correlation (cf Chapter 4) between accumulation of unrepaired nDNA damage and loss of neurons during aging may indicate that different types of neuron differ substantially in their cellular and molecular response to age-related stress, i.e. certain types of neuron seem to make use of distinct mechanisms to eliminate neurons with high amounts of nDNA damage during aging whereas other types of neuron do not. On the basis of recent studies indicating that the apoptotic response to nDNA damage is down-regulated in several organs during aging,⁵ it is tempting to hypothesize that the apoptotic response in the brain is likewise down-regulated, potentially in a cell type-specific manner. It will therefore be interesting to test this hypothesis in future studies by investigating different types of neuron for age-related changes in levels or activity of proteins involved in apoptosis. Furthermore, it appears important to identify cell type-specific differences in the molecular machinery responsible for the nDNA damage response and to identify target molecules for modulating these responses. Laser microdissection and microarray analysis^{6,7} of different types of neuron in the brain at various ages will be relevant for testing these hypotheses as these methodologies allow for the first time to compare gene expression among different types of neuron *in situ*.

Besides accumulation of nDNA damage, the aging brain is characterized by structural and functional alterations of synapses. Reductions in densities and numbers of synaptic terminals and spines preferentially occur during aging in neurons which form neocortical and hippocampal projections.⁴ Although unrepaired nDNA damage can lead to generation of altered proteins and proper function of various proteins is indispensable for normal synaptic function, little is known about the actual mechanisms connecting accumulation of nDNA damage with disturbed synaptic functions. In this respect strong evidence obtained from the aged human cerebral cortex indicated that age-related nDNA damage in promoter regions of genes reduced expression of genes with central roles in synaptic plasticity, vesicular transport and mitochondrial function.⁸ Oxidative stress selectively damaged the same gene promoters in cultured human neurons.⁸ Thus, nDNA damage induced by oxidative stress may selectively reduce the expression of genes responsible for normal synaptic function. A recent report in the literature suggested that increased levels of p53 may mediate effects of nDNA damage on synaptic integrity.⁹ Thus, it appears necessary to aim future investigations at the identification of preventable or recoverable molecular events associated with age-related nDNA damage and age-related disturbances in synaptic function and integrity.

Although a large number of studies have indicated that DNA mutations can result in age-related neurodegenerative disorders, evidence for a causative role of nDNA single strand breaks in neurodegeneration has been sparse. Very recently, however, a study has convincingly identified that nDNA single strand breaks can lead to neurodegenerative disease.¹⁰ In this study, defective repair of nDNA single strand breaks (caused by a mutation in tyrosyl phosphodiesterase 1) was shown to be the cause of spinocerebellar ataxia with axonal neuropathy.¹⁰ This defect in nDNA

repair resulted in a several-fold increase in the number of nDNA single strand breaks after free radical-induced nDNA damage.¹⁰ Further research is however warranted to assess putative effects of accumulation of nDNA single strand breaks on neuronal function during aging.

Caloric restriction and aging

My observations that caloric restriction reduced the relative level of nDNA damage in hippocampal neurons at high age and reduced age-related neuronal shrinkage indicate that caloric restriction influences important molecular events in the brain during aging. Ongoing studies will establish whether caloric restriction also reduced age-related synaptic alterations. Recent reports in the literature have shown that caloric restriction ameliorated damage of mitochondrial DNA,¹¹ resulted in enhanced expression of protein chaperones and neurotrophic factors,¹² and attenuated age-related decline in the rate of nDNA repair.¹³ Thus, accumulating evidence supports that caloric restriction potentially attenuates the aging process in the brain and several molecules involved in the beneficial actions of caloric restriction have recently been described.^{14–19} In addition, one could speculate that caloric restriction beneficially affects molecular and cellular mechanisms underlying neurodegenerative diseases. In this respect, a recent study indicated that caloric restriction reduced the burden of extracellular A β aggregates and the associated inflammatory reaction during aging in transgenic mice for both human mutant APP and human mutant PS1.²⁰ Nonetheless, detailed analyses with large controlled human trials involving long-term caloric restriction are necessary to actually determine the potential benefits of caloric restriction for humans.

Intraneuronal A β and neuronal loss in AD

There has been considerable discussion in the literature whether molecules proposed by the A β cascade hypothesis of AD pathogenesis result in age-related hippocampal neuronal loss *in vivo*. My findings indicated that extracellular A β deposits, high levels of intraneuronal A β and mutant PS-1 play significant roles in hippocampal loss of neurons and synapses (Figure 1). In addition, I have recently found that overexpression of human mutant APP in mice did not result in age-related loss of hippocampal neurons (unpublished observations)

With regard to neuron loss in AD, intraneuronal A β 42 is a major candidate. Abundant A β 42 was observed in pyramidal neurons in APP751^{SL}/PS-1^{M146L} mice before formation of extracellular A β deposits and was associated with markers of neuronal stress.^{21,22} Recent results from a novel transgenic mouse model of AD supported the notion that high levels of intraneuronal A β 42 in hippocampal pyramidal cells were connected with age-related loss of hippocampal neurons.²³

Thus from the candidates hypothesized to be responsible for age-related neuron loss in these transgenic mouse models (as explained in Chapter 1), intraneuronal A β and extracellular A β depositions have been identified as main players (Figure 1A). Future studies focusing on temporal and spatial expression and on dose effects of A β 42 may clarify the putative causal role of A β 42 in neuron loss in AD. Nonetheless, recent studies have provided evidence about the compartment of neurons in which A β is detectable in humans with AD and in animal models of

AD. A study in human postmortem materials described that A β immunoreactivity is localized in endolysosomal compartments of neurons in early stages in AD patients but not in aged people without memory complaints.²⁴ The level of intraneuronal A β immunoreactivity was reduced in advanced disease stages when abundant extracellular A β deposits were formed, suggesting that A β deposits may function as compact pools for A β generated intraneuronally.²⁴ A β immunoreactivity was primarily observed in types of neuron selectively affected in AD.²⁴ The presence of A β in neuronal endosomes and lysosomes in human brain supports previous *in vitro* work localizing APP, PS1 and A β to the secretory and endolysosomal system (as explained in Chapter 1 and illustrated in Supplementary Figure, see page 205). In APP751^{SL}/PS-1^{M146L} mice (i.e. the same mice as investigated by me in Chapter 8 and 9), A β was detected in endolysosomal compartments of cortical and hippocampal neurons similar to the human situation.²⁵ Similar to the temporal sequence in human AD, young APP751^{SL}/PS1^{M146L} mice showed higher levels of intraneuronal A β than older mice which have abundant extracellular A β deposits.²² The presence of A β in endolysosomes may have important effects for endolysosomal and cellular function²⁶ as disturbances of the endolysosomal system were selectively found in types of neuron prone for neurodegeneration in AD in the human brain^{27–29} as well as in APP/PS1 mice.³⁰ Besides the effects on the endolysosomal system, evidence is accumulating that A β and APP are associated with disturbances in axonal transport. Previous studies had already indicated that tau protein is critically involved in the regulation of axonal transport through its microtubule-binding function, and that hyperphosphorylation of tau as well as the formation of paired helical filaments are connected with a slowdown of axonal transport.³¹

Figure 4. Scheme summarizing major candidates for age-related loss of neurons (A) and synapses (B) in AD according to the A β cascade hypothesis. Note that PS1 mediates γ -secretase cleavage of C99 into A β (thin-lined arrow). Findings of this thesis have identified intraneuronal A β and extracellular A β as factors strongly connected with age-related loss of neurons (open arrows in A) whereas mutant PS-1 itself, mutant APP itself and the C99 terminal fragment of APP were shown not to be connected with age-related loss of neurons (crossed open arrows in A) in AD. Furthermore, mutant PS-1, intraneuronal A β and extracellular A β (open arrows in B) but not mutant APP itself and the C99 terminal fragment of APP (crossed open arrows in B) have been identified as factors strongly connected with age-related loss of synapses in AD.

In this respect recent evidence suggested that tau inhibits *in vitro* kinesin-I dependent transport of peroxisomes, neurofilaments, and Golgi-derived vesicles into neurites, particularly transport of APP, causing accumulation of APP in the neuronal cell body.³² Thus, regulation of axonal transport may substantially affect APP localization and processing. Axonal swellings filled with mitochondria, vacuoles, vesicles and kinesin were recently observed in the brains of transgenic APP mice well before any A β aggregations were formed³³ (note that the morphology and localization of these swellings resembled the enlarged SIPB I have observed in the surroundings of extracellular A β aggregates in APP751^{SL}/PS1^{M146L} and APP751^{SL} mice [cf. Chapter 9]). On the basis of the presence of kinesin in these swellings, Stokin and colleagues speculated that the APP transgenic mice they investigated had deficits in kinesin-mediated axonal transport.³³ By crossing the APP transgenic mice with kinesin-reduced mice, these authors found that reducing kinesin led to a decrease in anterograde transport of APP-Containing particles and to an increase of retrograde transport.³³ Thus, these findings indicated that APP mutations can reduce kinesin levels leading to changes in APP transport and formation of axonal swellings. In addition, the authors found evidence that kinesin-mediated reductions of axonal transport led to a selective increase in the ratio between A β 42 and A β 40, to increased intraneuronal accumulation of A β , and (later in life) to increased formation of extracellular A β aggregates. These findings indicate that mutations in APP alter axonal transport which in turn regulates APP localization and A β generation. Mutations in PS1 have furthermore been associated with alterations in kinesin-mediated axonal transport. It was recently shown that expression of the PS1 mutations M146V, I143T, and deletion of exon 9 (D9) in human neuronal cells *in vitro* significantly increased the activation of glycogen synthase kinase 3- β (GSK3- β).³⁴ *In vivo*, GSK3- β phosphorylates kinesin light chains and causes the release of kinesin-I from membrane-bound organelles, leading to a reduction in kinesin-I-mediated axonal transport.³⁵ Consistent with such a deficit in kinesin-I-mediated axonal transport, densities of synaptophysin-containing vesicles and mitochondria were found reduced in neuritic processes of hippocampal neurons expressing mutant PS-1^{M146V} *in vitro*.³⁴ In this respect, my observation of age-related loss of SIPB in PS-1^{M146L} and APP751^{SL}/PS-1^{M146L} transgenic mice supports a crucial role for disturbances of kinesin-mediated axonal transport in AD pathogenesis (Chapter 9). Taken together, these findings point to the endolysosomal system and kinesin-mediated axonal transport as crucial intraneuronal systems affected early in AD pathogenesis. Furthermore, they point to a crucial role of intraneuronal A β in AD pathogenesis. A fundamental current issue in the field is whether intraneuronal accumulation of A β is connected to cognitive impairments. Very recently, it was found in APP/PS1/tau transgenic mice that accumulation of intraneuronal A β accumulation is indeed associated with cognitive impairments (with initial disturbances in long-term retention).³⁶ Moreover, it was found that immunotherapy-mediated clearance of A β was able to reverse early disturbances in cognitive function.³⁶ It thus appears plausible that intraneuronal accumulation of A β is a key, potentially preventable, event in AD pathogenesis.

Many questions however await elucidation. What is the normal function of A β ? Why is A β generated at all during aging and why is it increased in AD? Is A β really the primary causative factor in AD pathogenesis, or is A β formed as a consequence to other alterations? Do endolysosomal disturbances lead to A β generation or does A β generation lead to endolysosomal disturbances? How is A β connected to abnormalities in tau proteins and vice versa? Does generation of intraneuronal A β occur in the same cells that harbor tau abnormalities in humans? In any

case, further detailed neurobiological research is highly warranted to answer these questions and to determine whether strategies that decrease A β levels in the brain will prevent or halt the pathogenesis of AD. In this respect, it is hopeful that implementation of several approaches in mouse models of AD has resulted in attenuation of neurodegeneration.^{37–40} Animal models of AD serve as an excellent framework to test hypotheses on these issues, to scrutinize the temporal sequence of events in AD and to determine the exact relationships between key molecules.

Protein aggregations in neurodegenerative diseases

Although my work mainly focused on intracellular and extracellular aggregations of A β protein as a culprit for neuronal loss in Alzheimer's disease, neuronal degeneration in AD is also associated with intracellular aggregations of another protein, i.e. hyperphosphorylated tau protein.⁴¹ Moreover, converging lines of evidence have revealed that many (if not all) age-related neurodegenerative diseases are characterized by aggregations and depositions of proteins.^{42–44} As such, Parkinson's disease is characterized by cytoplasmic Lewy bodies and aggregations of α -synuclein, Huntington's disease by nuclear and cytoplasmic inclusions, and amyotrophic lateral sclerosis by hyalinized inclusions of SOD1.⁴³ The identification of genetic mutations in familial forms of these diseases and the development of mouse models of these diseases have provided insights into the corresponding disease's pathogenesis. Based on these insights, it appears that these diseases share a common pathogenic mechanism: the generation of misfolded proteins that eventually accumulate and aggregate during aging.⁴⁴ It is known that the conformation and assembly of misfolded proteins generally follows certain transitions: misfolded proteins can form monomers, dimers, oligomers and eventually fibrils.⁴³ Misfolded proteins are normally recognized by chaperone proteins, transported and degraded by the proteasome or phagolysosomes. However, when these systems become inefficient or overwhelmed, misfolded proteins accumulate within cells. Depending upon the nature of the proteins and the cell itself, misfolded proteins can be excreted and may aggregate extracellularly or can aggregate intracellularly.⁴³ It is currently a matter of scientific debate in which conformational state misfolded proteins are most toxic. In regard of A β protein, recent evidence suggested that protofibrillar conformational states of A β can associate with APP and form pores in cellular membranes.⁴⁵ In addition, intraneuronal soluble A β oligomers have been found to affect synaptic integrity.⁴⁶ Recent work suggested that intraneuronal A β levels decrease as a result of extracellular deposition of fibrillar A β .^{22,24} Thus, one could speculate that aggregation of A β peptides in the form of extracellular depositions of fibrillar A β may be an escape mechanism for pooling excess amounts of intraneuronal A β peptides. Recent developments in other neurodegenerative disease support that neurodegeneration and neuronal loss depend on the localization and conformational state of misfolded proteins.^{42,47} One could therefore hypothesize that attenuation of neuronal loss in various age-related neurodegenerative diseases may be reached via enhancement of molecules with chaperoning and degrading functions and via modulation of conformational states of misfolded proteins.

In conclusion, despite the fact that our knowledge on how the brain changes during aging and in AD is still rather limited, considerable progress has been made over the last years in understanding some of the molecular and cellular events underlying neuronal loss in aging and AD. The identification of several preventable or reversible events offers hope for rational intervention.

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Summary

To prevent or treat neuronal alterations and neuronal loss in aging and Alzheimer's disease (AD), it is crucial to identify the underlying molecular and cellular mechanisms. Although our knowledge about these mechanisms is still rather limited, recent findings have provided novel insights and envision potential candidates for intervention. The findings of my thesis have contributed to this development. In **Chapter 1**, I describe background information on the current knowledge on the aging process in the brain and on the molecular and cellular events that are involved in the pathogenesis of AD. In addition, I discuss current issues on the mechanisms underlying neuronal loss in aging and AD and on potential strategies to attenuate these mechanisms. Based on these issues, I have formulated several hypotheses which were tested in my PhD research. The studies which tested my hypotheses are described in Chapters 2 to 9.

A major prerequisite for performing adequate investigations is that the methodology used is appropriately measuring the parameter under investigation. In **Chapter 2**, I describe that quantitative neuromorphologic analyses on the brain should be carried out on sets of sections systematically and randomly sampled to span the entire brain region of interest and not on single sections. These findings resulted into new standards for quantitative neuromorphological investigations, based on which I carried out all further investigations. In **Chapter 3**, I speculate (*on the basis of a review of the literature*) on the existence of a potential, previously unknown, inverse association in the aging brain between types of neuron showing a accumulation of nuclear (n) DNA damage and types of neuron showing an age-related reduction in numbers. This speculation was tested for the first time in a controlled study (described in Chapter 4) on one mammalian species, i.e., a cohort of aging mice of a specific strain. In **Chapter 4**, I describe this study in which I showed that types of neuron in the mouse brain with age-related decline in the rate of nDNA repair showed accumulation of unrepaired nDNA damage during aging without age-related loss of neurons. In contrast, types of neuron without an age-related decline in the rate of nDNA repair showed no accumulation of unrepaired nDNA damage during aging but an age-related loss of neurons. These findings indicate that the aging process in the brain affects certain types of neuron differently than others. In addition, these findings suggested that attenuation of age-related accumulation of nDNA damage in neurons may be reached through several strategies.

One of the best documented sources for damage during aging is oxidative stress (the increased production of highly reactive oxygen molecules). In **Chapter 5**, current evidence for a central

role of oxidative stress in Alzheimer's disease and for the value of antioxidants (molecules that scavenge these reactive molecules) in preventing or treating Alzheimer's disease is critically discussed. In **Chapter 6**, I describe the study which was designed to investigate the effects of an upregulation of an endogenous antioxidant (through transgenic overexpression of superoxide dismutase) and of a reduction in dietary caloric intake. The effects were studied by investigating the amount of DNA damage in neurons and on the total number and size of neurons in the hippocampus during aging. I chose to investigate the hippocampus as this brain region is known to be highly vulnerable during aging. I found that life-long upregulation of superoxide dismutase did not have any effect on the amount of DNA damage in neurons and on the total number and size of neurons in the hippocampus during aging. In contrast, a dietary reduction in caloric intake resulted in an extension of life span, a reduction in the amount of DNA damage, a reduced volume of the hippocampus, and attenuation of neuronal shrinkage during aging.

Although AD is the most common age-related mental health disorder, very little is known about the molecular and cellular events underlying neuronal alterations in AD. The most striking neuronal alterations in AD are loss of neurons and synapses. So far, there is no available treatment which modifies the molecular and cellular processes involved in AD pathogenesis. Several candidate molecules have recently been proposed to be responsible for loss of neurons and synapses in AD. The ' β -amyloid-hypothesis' is the most commonly accepted hypothesis. This hypothesis poses that aggregations of β -amyloid protein result into damage in the brains of people with Alzheimer's disease. Loss of neurons and synapses (connections between neurons) are the most important forms of damage and these parameters can be investigated with modern techniques. A way to investigate numbers of synapses is quantification of the number of synaptophysin-immunoreactive presynaptic boutons [SIPB]). Transgenic animals (animals whose DNA is altered so that they produce a certain molecule in a higher amount than normal animals do) are very useful to investigate the effects of these molecules on living organisms. With the help of several transgenic mouse models, I have rigorously assessed the effects of several of the proposed candidates on the numbers of neurons and synapses in the hippocampus during aging.

In **Chapters 7, 8 and 9** I describe that

- a) overexpression of the C99 terminal fragment of human Amyloid Precursor Protein (APP) did not result in age-related loss of hippocampal neurons or synaptophysin-immunoreactive presynaptic boutons within the hippocampus,
- b) overexpression of human mutant APP did not result in age-related loss of SIPB in the hippocampus,
- c) overexpression of human mutant Presenilin 1 (PS1) resulted in age-related loss of SIPB in hippocampal area CA1-2 but not in age-related loss of hippocampal neurons,

- d) overexpression of both human mutant APP and human mutant PS1 resulted in age-related formation of extracellular A β aggregations, which were associated with age-related hippocampal neuron loss and with disturbances of synaptic integrity,
- e) mechanisms other than extracellular A β aggregations contributed significantly to age-related hippocampal neuron loss in mice that overexpress both human mutant APP and human mutant PS1.

Together, these findings have prompted for modifications of the original 'β-amyloid hypothesis' as they indicated that certain molecules are much more likely to be held responsible for loss of neurons and synapses in the hippocampus of patients with AD than other molecules. By defining the molecules responsible for neuronal loss, these findings may initiate the development of novel treatment strategies to prevent or treat this devastating disease. In **Chapter 10**, I summarize the major results of my thesis and discuss them in light of the current literature. Furthermore, possible avenues for future research are discussed.

Samenvatting

Om veranderingen en verlies van materiaal in zenuwcellen (i.e. neuronen) tijdens veroudering en bij de ziekte van Alzheimer te voorkomen of te behandelen, is het cruciaal om de onderliggende moleculaire en cellulaire mechanismen te identificeren. Ondanks de beperkte kennis over deze mechanismen, hebben recente bevindingen geleid tot nieuwe inzichten en liggen er potentiële kandidaten voor interventie in het verschiet. De bevindingen zoals beschreven in dit proefschrift hebben bijgedragen aan deze ontwikkeling. In **Hoofdstuk 1** beschrijf ik achtergrond informatie over de huidige kennis van het verouderingsproces in de hersenen en van de moleculaire en cellulaire gebeurtenissen die betrokken zijn bij de pathogenese van de ziekte van Alzheimer. Verder bespreek ik huidige discussie punten over de mechanismen die ten grondslag liggen aan neuronaal verlies bij veroudering en bij de ziekte van Alzheimer. Met deze punten als uitgangsbasis heb ik verschillende hypothesen geformuleerd die ik tijdens mijn promotieonderzoek heb getest. In Hoofdstukken 2 tot en met 9 bespreek ik de studies waarin ik de hypothesen heb getest.

Een belangrijke voorwaarde om adequaat onderzoek te doen, is een correcte meetmethode om de parameter van interesse op een juiste manier te onderzoeken. In **Hoofdstuk 2** beschrijf ik dat kwantitatieve neuromorfologische meetmethoden voor het brein moet worden uitgevoerd met series van hersencoupees die op een systematische doch willekeurige manier worden verzameld en die de gehele hersenregio onder analyse omvatten, en niet met een enkele hersencoupe door die hersenregio. Op basis van deze bevinding heb ik al mijn verdere neuromorfologische onderzoeken uitgevoerd met series van hersencoupees die op een systematische doch willekeurige manier werden verzameld en die de gehele hersenregio onder analyse omvatten. In **Hoofdstuk 3** speculeer ik (op basis van een analyse van de literatuur) over het mogelijke bestaan van een vooralsnog onbekende, omgekeerde associatie in de hersenen tussen typen neuronen die DNA schade ophopen in de celkern tijdens veroudering en typen neuronen die verminderen in aantal tijdens veroudering. Deze speculatie werd voor de eerste keer getest in een gecontroleerde studie met één diersoort, muizen van een bepaalde stam. In **Hoofdstuk 4** beschrijf ik deze studie waarin ik heb aangetoond dat, tijdens het verouderingsproces van deze muizen, de typen neuronen met een vermindering van de snelheid van DNA reparatie een ophoping van ongerepareerde DNA schade hadden in hun celkern zonder dat het totale aantal neuronen significant verminderde. Andersom had een ander type neuron geen vermindering van de snelheid van DNA reparatie en geen ophoping van ongerepareerde DNA schade in de celkern maar wel een significante vermindering van het aantal neuronen. Deze bevindingen laten zien

dat het verouderingsproces bepaalde typen neuronen anders beïnvloedt dan andere. Eén van de best gedocumenteerde bronnen voor schade tijdens veroudering is oxidatieve stress (i.e. de verhoogde productie van snel-reactieve zuurstof moleculen). In **Hoofdstuk 5**, bespreek ik de centrale rol van oxidatieve stress bij de ziekte van Alzheimer en de waarde van antioxidanten (i.e. moleculen die deze snel-reactieve moleculen kunnen wegvangen) om de ziekte van Alzheimer te voorkomen of te behandelen. In **Hoofdstuk 6** beschrijf ik de studie die ik heb uitgevoerd om de effecten van een verhoging van een endogene antioxidant (door transgene overexpressie van superoxide dismutase) en van een beperking van calorieën in de voeding op de hoeveelheid DNA schade in neuronen en op het totale aantal en de grootte van neuronen in de hippocampus tijdens veroudering te onderzoeken. De hippocampus werd gekozen omdat deze hersenregio erg kwetsbaar is tijdens veroudering. Ik heb gevonden dat levenslange verhoging van superoxide dismutase geen effect had op de hoeveelheid DNA schade en op het totale aantal en de grootte van neuronen in de hippocampus tijdens veroudering. Tegengesteld hieraan resulteerde een beperking van calorieën in de voeding tot een verlenging van de levensduur, een vermindering van de veroudering-gerelateerde ophoping van DNA schade, een vermindering van het volume van de gehele hippocampus en een mindere veroudering-gerelateerde verkleining van neuronen. Dus, beperking van calorieën heeft meer effecten op de gemeten veroudering-gerelateerde parameters dan de verhoging van het endogene antioxidant.

Ondanks dat de ziekte van Alzheimer de meest voorkomende veroudering-gerelateerde stoornis is van de geestelijke gezondheid, is slechts weinig bekend over de moleculaire en cellulaire gebeurtenissen die ten grondslag liggen aan de neuronale veranderingen bij de ziekte van Alzheimer. Tot nu toe is er geen behandeling beschikbaar die de moleculaire en cellulaire gebeurtenissen die in de pathogenese van de ziekte van Alzheimer betrokken zijn, verandert. De laatste jaren werden verschillende kandidaat moleculen als verantwoordelijk gesteld voor de schade in de hersenen bij mensen met de ziekte van Alzheimer. De 'β-amyloid-hypothese' is de meest gangbare hypothese. Deze stelt dat ophopingen van het β-amyloid eiwit leiden tot schade in de hersenen bij mensen met de ziekte van Alzheimer. Verlies van neuronen en van synapsen (verbindingspunten tussen neuronen) zijn hierbij belangrijke vormen van schade. Deze parameters kunnen worden onderzocht met moderne meettechnieken. Een methode om het aantal synapsen vast te stellen is het quantificeren van het aantal synaptophysine-immunoreactieve presynaptische boutons (SIPB). Met gebruik van transgene dieren (dieren waarvan het DNA zodanig gemanipuleerd is dat ze een bepaald molecuul meer aanmaken dan normale muizen) is het mogelijk om de effecten van (over)expressie van bepaalde moleculen op levende organismen te bestuderen. In mijn promotieonderzoek heb ik de effecten onderzocht van verschillende van de voorgestelde kandidaat-moleculen op het aantal neuronen en synapsen in de hippocampus tijdens veroudering van deze dieren:

In **Hoofdstukken 7, 8 en 9** beschrijf ik dat:

- a) transgene overexpressie van het C99-terminale fragment van het humane amyloid-precursor-protein (APP) geen verlies van hippocampale neuronen of SIPB tot gevolg had,

- b) transgene overexpressie van humaan gemuteerd APP niet leidde tot verlies van SIPB in de hippocampus tijdens veroudering,
- c) transgene overexpressie van het humane gemuteerde preseniline 1 (PS1) leidde tot verlies van SIPB in een speciaal gebied (het CA1–2 gebied) van de hippocampus maar niet tot verlies van hippocampale neuronen tijdens veroudering,
- d) transgene overexpressie van het humane gemuteerde APP en PS1 leidde tot de vorming van extracellulaire ophopingen van het β -amyloid eiwit, die verband hielden met verlies van neuronen, verstoringen van de synaptische integriteit en verlies van SIPB in de hippocampus tijdens veroudering
- e) andere mechanismen dan de extracellulaire ophopingen van β -amyloid significant bijdroegen aan verlies van neuronen in de hippocampus tijdens veroudering van transgene muizen voor APP en PS1.

Gezamenlijk hebben mijn bevindingen uitgewezen dat verlies van neuronen en synapsen in de hippocampus bij mensen met de ziekte van Alzheimer waarschijnlijk wordt veroorzaakt door bepaalde moleculen en hoogst waarschijnlijk niet door andere, hetgeen tot modificaties van de originele ' β -amyloid hypothese' heeft geleid. Bovendien kunnen mijn bevindingen fungeren als een basis voor de ontwikkeling van nieuwe strategieën om deze verwoestende ziekte te voorkomen of te behandelen. In *Hoofdstuk 10* vat ik de voornaamste bevindingen van mijn promotieonderzoek samen en bediscussieer ik deze in het licht van de huidige literatuur. Verder bespreek ik mogelijke richtingen voor toekomstig onderzoek.

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Curriculum vitae

Bart Rutten was born on May 21st 1975 in Sittard. He graduated for secondary school, VWO, at Serviam Lyceum in Sittard in 1993. From 1993 until 1994, he studied medicine at Catholic University Leuven (Belgium). In 1994, he continued his medical training at Maastricht University. The author received his doctoral diploma in medicine with first class honours in 1998. After a research internship at University of California at San Francisco (UCSF, USA), an elective internship in pediatrics at Frere Hospital in East London (South Afrika) and experimental research at the division of Cellular Neuroscience (Department of Psychiatry and Neuropsychology), he received his medical doctor degree with first class honours in 2000. The author started with his Ph.D. research on neuronal loss in aging en Alzheimer's disease with dr. C. Schmitz and prof. dr. HWM Steinbusch, division Cellular Neuroscience in 2001. In the framework of his PhD research, the author was granted a Marie-Curie scholarship of the European Union for a one year research period with prof. dr. H Korr, Department of Anatomy and Cell Biology at RWTH University in Aachen (Germany). In addition, the author received grants from the Dutch Scientific Organization (NWO) for a working visit with prof. dr. DC Wallace at the Center for Molecular Medicine at Emory University in Atlanta (Georgia, USA), from the Ellison Medical Foundation for attendance of the course Molecular Biology of Aging in Woods Hole (Massachussets, USA), from the International Alzheimer's Disease Association for a working visit at MBF Inc. in Burlington (Vermont, USA) and travel grants for the Human Brain Conference (Rome, Italy), and the yearly conferences of the Society of Neuroscience in the USA. Furthermore, he has been assistant-editor of the Journal of Chemical Neuroanatomy and advisor of the Committee of Reseach Management at Maastrich University for a period of one year. The results of his PhD research were described in this thesis. In April 2005, the author started his residency in Psychiatry at the Academic Hospital Maastricht and Psycho Medisch Streekcentrum Vijverdal, headed by prof. dr. J van Os and dr. K. Schruers.

Curriculum vitae

Bart Rutten werd geboren op 21 mei 1975 in Sittard. In 1993 behaalde hij het eindexamen VWO aan het Serviam Lyceum in Sittard. Van 1993 tot 1994 studeerde hij geneeskunde aan de Katholieke Universiteit Leuven (België). In 1994 startte hij met de studie geneeskunde aan de Universiteit Maastricht. In 1998 behaalde hij zijn doctoraal diploma geneeskunde cum laude. Na een wetenschapsstage aan de Universiteit van Californië te San Francisco (UCSF, Verenigde Staten van Amerika), een keuze coschap Kindergeneeskunde bij het Frere Hospital te East London (Zuid Afrika) en onderzoek bij de divisie Cellulaire Neurowetenschappen (vakgroep Psychiatrie en Neuropsychologie), behaalde hij in 2000 zijn arts diploma cum laude. In 2001 is de auteur begonnen met zijn promotieonderzoek naar veroudering en de ziekte van Alzheimer bij dr. C Schmitz en prof. dr. HWM Steinbusch, divisie Cellulaire Neurowetenschappen. In het kader hiervan heeft hij een Marie-Curie beurs gekregen van de Europese Unie om gedurende 1 jaar onderzoek te verrichten bij prof. dr. H Korr, vakgroep Anatomie en Cellulaire Biologie aan de RWTH Universiteit in Aken (Duitsland). Verder verkreeg de auteur beurzen van de Nederlandse Wetenschappelijke Organisatie (NWO) voor een werkbezoek in het kader van zijn promotieonderzoek bij prof. dr. DC Wallace in het Center for Molecular Medicine aan de Emory University in Atlanta (Georgia, USA), van de Ellison Medical Foundation voor de cursus Molecular Biology of Aging in Woods Hole (Massachusetts, USA), van de Internationale Stichting Alzheimer Onderzoek voor een werkbezoek aan MBF Inc. in Burlington (Vermont, USA), en reisbeurzen voor de Human Brain Conference (Rome, Italië) en de jaarlijkse congressen van de Society of Neuroscience in de Verenigde Staten. Tevens was hij assistent-editor van het tijdschrift Journal of Chemical Neuroanatomy en adviseur van de Commissie Onderzoeks Beleid van de Universiteit Maastricht gedurende 1 jaar. De resultaten van zijn promotieonderzoek heeft u kunnen lezen in dit proefschrift. In april 2005 startte de auteur met de opleiding psychiatrie van het Academisch Ziekenhuis Maastricht en het Psycho Medisch Streekcentrum Vijverdal, bij opleiders prof. dr. J van Os en dr. K. Schruers.

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Supplementary figure. Illustrations depicting generation and localization of intraneuronal β -amyloid ($A\beta$). Amyloid Precursor Protein (APP, red structure with two tails) is localized in membranes and can be cleaved by β -secretase and subsequently by γ -secretase into $A\beta$. These cleavage steps (depicted by green arrows) may occur in the cellular membrane (as shown in the left panel) but may also occur in membranes of structures belonging to the Golgi network and endolysosomal system (right panel). Intracellular $A\beta$ may form dimers and oligomers intraneuronally and may be secreted into the extracellular matrix where $A\beta$ may furthermore aggregate in the form of plaques (see left panel).

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