Aus der Klinik für Psychiatrie und Psychotherapie
(Prof. Dr. med. J. Wiltfang)
der Medizinischen Fakultät der Universität Göttingen

Quantitative analysis of neuropathological alterations in two transgenic mouse models of Alzheimer's disease

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Anastasiia Kurdakova

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Dekan:	Prof. Dr. rer. nat. H. K. Kroemer
Referent/in:	PD. Dr. rer. nat. O. Wirths
Ko-Referent/in:	Prof. Dr. Inga Zerr

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Affidavit:

I hereby declare that my doctoral thesis entitled ***"Quantitative analysis of neuropathological alterations in two transgenic mouse models of Alzheimer's disease"*** has been written independently with no other sources and aids than quoted.

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5XFAD	transgenic mice (Tg6799) expressing five familial AD mutations
$5XFAD_{hem}$	hemizygous 5XFAD
$5XFAD_{hom}$	homozygous 5XFAD
ABC	avidin-biotin complex
AD	Alzheimer's disease
AICD	APP intracellular domain
ANOVA	analysis of variance
APP	amyloid precursor protein
asf	area sampling fraction
Aβ	amyloid beta
bp	base pair(s)
CA	cornu ammonis
CNS	central nervous system
CR	cognitive reserve
DAB	3,3'-Diaminobenzidin
DCX	doublecortin
ddH_2O	distilled, deionized water
DG	dentate gyrus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EE	enriched environment
EOAD	early-onset Alzheimer's disease
FAD	familial Alzheimer's disease
FCS	fetal calf serum
GCL	granular cell layer
H_2O_2	hydrogen peroxide
hAPP	human APP
HCl	hydrochloric acid
hem	hemizygous
hom	homozygous
IVC	individually ventilated cages
LOAD	late-onset Alzheimer's disease
mAPP	murine APP
MCI	mild cognitive impairment
$MgCl_2$	magnesium chloride

m	month
min	minute
n	number of animals
NaCl	sodium chloride
NFT	neuro-fibrillary tangles
NIA	National Institute of Aging
NIH	National Institutes of Health
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pGlu	pyroglutamate
PSEN	presenilin
qRT-PCR	real-time quantitative PCR
rpm	revolutions per minute
RT	room temperature
SAD	sporadic Alzheimer's disease
SDS	Sodium dodecyl sulfate
SGZ	subgranular zone
SH	standard housing
ssf	section sampling fraction
T	mean section thickness
Tg4-42	transgenic mice overexpressing Aβ4-42
$Tg4\text{-}42_{hom}$	homozygous Tg4-42
TRH	thyreotropin releasing hormon
Tris	Tris (hydroxymethyl) aminomethane
tsf	thickness sampling fraction
WHO	The World Health Organization
WT	wild-type C57B6/J
Z	dissector height

1. INTRODUCTION

1.1 Dementia

Dementia is defined as “a progressive organic mental disorder characterized by chronic personality disintegration, confusion, disorientation, stupor, deterioration of intellectual capacity and function, and impairment of control of memory, judgment, and impulses” (Mosby’s medical dictionary 2009, p. 501). Consciousness is usually not affected. Although dementia mainly affects older people, it is not considered to be a normal part of ageing. Dementia is characterized by “inexorably progressive deterioration in cognitive ability and capacity for independent living” (Prince et al. 2013, p. 64).

There are many causes of dementia. Some of them are drug or alcohol intoxication, pernicious anaemia, hyperthyroidism, paresis, subdural hematoma, benign brain tumour, hydrocephalus, insulin shock, or tumour of islet cells of the pancreas. These dementias can be reversed by treating the underlying condition. Quite the contrary, Alzheimer's disease, Pick's disease, vascular dementia, Lewy body dementia and other organic forms of dementia are generally considered irreversible, progressive, and incurable (Mosby’s medical dictionary 2009).

Alzheimer’s disease, vascular dementia, Lewy body dementia, and frontotemporal dementia are the most common underlying pathologies of dementia (Prince et al. 2013). Among these four pathologies, Alzheimer's disease is the most common cause of dementia and may contribute to 60–70% of cases (World Health Organization 2015).

1.1.1 Epidemiology of dementia

Dementia is a very burdensome condition and is a top cause of disability and dependency among older people worldwide. It has an enormous physical, psychological, social and economic impact on caregivers, families and the society.

According to The World Health Organization (March 2015), 47.5 million people worldwide have dementia and there are 7.7 million new cases every year. The World Alzheimer Report 2015 states that these numbers will almost double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050. These new estimates are 12-13% higher comparing to the estimates from the World Alzheimer Report 2009. The prevalence of dementia in people aged 60 years and older ranges from 4.6% in Central Europe to 8.7% in North Africa and the Middle East. Rapidly increased prevalence of chronic diseases like dementia is associated with increasing life expectancy. Over the last century life expectan-

cy increased from an average of 47 and 49 to 75 and 80 years of age for men and women respectively (Anderson and DeTurk 2002). Nowadays, there are almost 900 million people aged 60 years and older living worldwide (World Alzheimer Report 2015). In most countries, the rise in life expectancy is accompanied by declining fertility rates, so that older people also constitute an increasing proportion of total population (NIH, National Institute of Aging 2011).

1.1.2 The topicality of a problem of dementia and the importance of the ongoing research in this field

The global cost of Alzheimer's disease and dementia is estimated to be US$ 818 billion in 2015, which is equivalent to 1.09% of the entire world's gross domestic product. It is an increase of 35.4% comparing to 2010 (US$ 604 billion in 2010) (World Alzheimer Report 2015).

In the context of the rapidly unfolding demographic changes, dementia becomes a health- and social-care priority for many countries. Governments in such high-income countries as the UK, France, Norway, USA, and South Korea are developing and implementing specific plans or strategies (Prince et al. 2013). However, in the World Alzheimer Report 2015 it was estimated that 58% of all people with dementia live in countries currently classified by the World Bank as low- or middle-income countries. These countries have fewer economic and professional resources to face the health and social care needs of their aging populations (World Alzheimer Report 2015). Particularly rapid increases in the numbers and proportion of older people are forecast for China, India, and Latin America (Prince et al. 2013).

The topic "Dementia" is also very present in mass media and arts. People search for the information about dementia, because they are afraid of it. They forced to deal with this problem, because they themselves, their close relatives or friends are affected (Peel 2014). Besides numerous articles in mass media, reports of the WHO, international and local Alzheimer's societies, many books were written and movies produced that deal with this problem. Among them are the American drama film „Still Alice" (2014) based on Lisa Genova's 2007 novel of the same name, the German tragicomedy film "Head Full of Honey" (2014), the American science fiction film "Rise of the Planet of the Apes" (2011).

For all these reasons, it became important to intensify the research in the field of dementia and Alzheimer's disease in particular. In the last few decades, numerous substantial results in the field of Alzheimer's disease were achieved. The dominant familial Alzheimer (FAD) mutations, genetic and environmental risk factors for sporadic AD were identified, different transgenic animal models were created (Woodruff-Pak 2008), the drugs that can slow down the evolution of symptoms were developed (Rafii and Aisen 2009), the endogenous neurotoxic species were characterised and the subsequent clinical trials of immunization against them were performed (Kayed and Jackson 2009; Wisniewski and Konietzko 2008). It was also shown that a modulation of environmental factors and improving physical conditions has been efficient in the prevention of AD. Understanding the effects of lifestyle factors on the disease course might help directing therapeutic approaches (Arenaza-

Urquijo et al. 2015). Moreover, some epidemiological studies have shown that higher education levels could postpone the onset of the dementia syndrome (Paradise et al. 2009) and act as protective factors against the cognitive symptoms of AD (Roe et al. 2007). The underlying mechanism of this phenomenon is supposed to be a so called ***cognitive reserve*** (Arenaza-Urquijo et al. 2015). The cognitive reserve implies that persons with more cognitive capacities can cope longer and more effective with the pathological manifestations of AD due to a higher variability at the level of brain networks (Stern 2002; Stern 2006; Stern 2009).

The lifestyle-based interventions and increasing intellectual capacities by cognitive and physical exercise are potential low-cost preventive measures that could delay the onset of AD and help maintaining cognitive performance of the patients.

1.2 Alzheimer's disease: history and epidemiology

In 1906, at the 37th meeting of the Society of Southwest German Psychiatrists in Tübingen, the German psychiatrist Alois Alzheimer reported about one peculiar case of a patient, the 51 years-old Auguste Deter, from the Frankfurt Asylum. He observed this patient from 1901 till her death in 1906 (Goedert and Spillantini 2006). Auguste Deter suffered from cognitive and language deficits like memory disturbance, loss of orientation, delusions, auditory hallucinations as well as aggressive behaviour (Alzheimer 1907). After her death, Alzheimer took the patient's brain to Munich and investigated it using staining technics in Emil Kraepelin's lab. Together with other scientist of Kraepelin's lab, Alois Alzheimer identified macro- and microscopic alterations such as brain atrophy, amyloid plaques and neurofibrillary tangles. The case of Auguste Deter reported on the 3rd November 1906 in Tübingen was the first reported case of the disease with this characteristic combination of symptoms, and the first time that the pathology and the clinical symptoms of presenile dementia were presented together (Maurer and Maurer 2003). In his textbook in 1910, Emil Kraepelin coined the term "Alzheimer's disease" (Kraepelin 1910) and thereby established the name, which is still used today.

Alois Alzheimer died only 9 years after he described the case of Auguste Deter (Maurer and Maurer 2003). He might have hardly imagine that 100 years later the most common neurodegenerative disease would be called after him, and speaking about dementia we would mostly speak about Alzheimer's disease.

Alzheimer's disease is the most common cause of dementia with nearly 30 million people worldwide suffering from it (according to the WHO 2015). Currently, 1.5 million people suffer from dementia in Germany. According to German Alzheimer's Association (Deutsche Alzheimer Gesellschaft), almost two-thirds of them are suffering from Alzheimer's disease. Due to the ageing population, the number of dementia patients increases continuously, with around 300000 new patients every year. Unless there will be a decisive breakthrough in prevention and therapy, the number of people affected by dementia in Germany will increase, reaching 3.0 million in 2050 (Deutsche Alzheimer Gesellschaft 2014).

1.3 Clinical aspects of Alzheimer's disease

1.3.1 Progression of disease

The progression of Alzheimer's disease (AD) is slow and results in a progressive decline of memory functions in combination with non-cognitive symptoms like personality and mood changes finally leading to inability to perform everyday activities (Alzheimer´s Association 2012). The average development from mild and moderate AD to severe AD occurs in the average time of 7 to 10 years (Alzheimer's Association 2012; Holtzman et al. 2011). Early-onset AD happens to people who are younger than age 65. Often, they are in their 40s or 50s when AD is diagnosed in them. Most patients suffering from the early-onset AD have familial AD (an inherited form, see 1.3.2). However, the majority of AD cases are referring to late-onset AD, which occurs sporadically in people being 65 years and older. Late-onset AD arises from a complex of brain changes and impairments that occur over decades (NIH, National Institute of Aging 2015).

In 2011, the Alzheimer's Association and the National Institute on Aging (NIA) proposed new guidelines for the classification and diagnosis of AD. The course of AD was divided in three stages: ***preclinical AD***, ***mild cognitive impairment (MCI)*** due to AD and ***dementia*** due to AD (Albert et al. 2011; Jack et al. 2011; McKhann et al. 2011; Sperling et al. 2011). Preclinical AD is a newly defined stage of the disease. It reflects the evidence that measureable biomarker changes in the brain may occur years before symptoms affecting memory, behaviour or thinking can be detected. It was estimated that neurodegeneration and changes in brain start already 20 years before AD can be diagnosed (Blennow et al. 2006). During the preclinical phase, patients do not have any measurable clinical symptoms, whereas individuals with mild cognitive impairment show a visible decline compared to same-aged healthy individuals (Alzheimer's Association, 2012). The conversion of MCI cases into dementia due to AD occurs each year with a rate of 10-15%. In these cases MCI is considered to be an early stage of AD (Petersen 2004; Visser et al. 2005). Later, patients develop dementia due to AD. This stage is characterized by severe memory decline, language dysfunction, personality and behaviour changes, motor impairments and loss of visuospatial abilities (Alzheimer's Association 2012; Holtzman et al. 2011).

AD affects each person in a different way, also depending upon the person's personality before acquiring the disease. The progression of disease can be divided in three stages. The onset of the ***early stage*** of AD is gradual and can often be overlooked. The common symptoms are forgetfulness, losing track of time and becoming lost in formerly familiar places. At the ***middle stage***, the symptoms are clearer and more restricting. These can include regularly forgetting recent events, names and faces, becoming increasingly repetitive, getting lost at home, and having problems finding the right words. Mood and behaviour changes such as apathy and irritability occur. Patients lose confidence and need help with personal care. At the ***late stage*** of AD, patients are inactive and totally dependent on caregivers. Memory disturbances are severe and physical symptoms are obvious. At this stage, symptoms include being unaware of the time and place, having difficulty recognizing relatives and friends, having an increased need for assisted self-care, having difficulty

walking, becoming unsteady on the feet and falling down, showing behaviour changes that may escalate and include aggression. Moreover, patients can become sad or depressed. At the late stage, people may also experience hallucinations. Anxieties or phobias are also common (World Health Organization 2015).

At the terminal stage of the disease patients are even unable to perform basic motor functions like swallowing. This accumulation and progressive severity of symptoms is ultimately fatal and finally leads to death. Although dementia is a dramatically life-shortening illness, other conditions or illnesses may actually cause a person's death. For example, pneumonia is listed as the ultimate cause of death in up to two-thirds of people with dementia (Holtzman et al. 2011; Wada et al. 2001).

1.3.2 Genetic factors

As mentioned above, there are two forms of AD described: a familial AD and a sporadic AD. A familial AD (FAD) is an inherited form and constitutes approximately 1% of the disease cases (Zetterberg and Mattsson 2014). The majority of cases (99%) is classified as sporadic AD (SAD).

Persons originating from families, members of which have had early-onset AD (EOAD) over at least two generations, have a high risk of developing FAD (Selkoe 2001). Familial AD typically develops before the age of 65 years (early-onset) and is caused in the first place by overproduction of Abeta-protein (Aβ) due to mutations either in the amyloid precursor protein (APP) gene or genes encoding presenilin 1 (PSEN1) or presenilin 2 (PSEN2). Approximately 50% of the FAD patients carry mutations in one of the three genes PSEN1, PSEN2, or APP (Shea et al. 2015). PSEN1, PSEN2 and APP are located on the chromosome 14, 1 and 21, respectively (Masuhr and Neumann 2007; Rogaev et al. 1995; Sherrington et al. 1995).

PSEN1 and PSEN2 are essential components of the γ-secretase complexes. γ-secretase is responsible for cleavage and release of Aβ peptides of various lengths (see 1.6.2.2). In their non-mutated forms, presenilins play a variety of physiological roles such as cell proliferation (Wines-Samuelson and Shen 2005), cellular differentiation (Baumeister 1999), and neurotransmitter release (Zhang et al. 2009a).

The first described causes of FAD were mutations in the APP gene (Goate et al. 1991). APP mutations are located close to the secretase cleavage sites. For example, the first described APP mutation, London mutation APP_{V717I}, is located on the γ-secretase cleavage site. This mutation replaces valine with isoleucine at protein position 717. This mutation increases the Aβ42/Aβ40 ratio by increasing Aβ42 levels, while Aβ40 levels are not significantly affected (Eckman et al. 1997; Jonghe et al. 2001; Theuns et al. 2006; Herl et al. 2009).

The Swedish mutation $APP_{K670N, M671L}$ is the only known mutation near the β-secretase cleavage site. It is a double mutation, resulting in a substitution of two amino acids, lysine and methionine to asparagine and leucine, respectively. This well-known mutation was found in two large Swedish families, which were connected genealogically. Affected indi-

viduals presented first with memory loss and all met diagnostic criteria for AD (Mullan et al. 1992). The Swedish mutation increases the total Aβ levels (Haass et al. 1995).

Another APP mutation, APP_{I716V} or so-called Florida mutation was first reported in an American patient with suspected Alzheimer's disease. She had a family history of dementia with a mean age of onset of about 53 years (Eckman et al. 1997).

Another form of the APP-associated AD has been observed in patients with Down's syndrome. An increased Aβ peptide production is caused by an increased dosage of chromosome 21 genes. The plasma levels of amyloid beta proteins $Aβ_{1-40}$ and $Aβ_{1-42(43)}$ are both elevated in Down's syndrome (Tokuda et al. 1997).

Even though, the autosomal dominant forms of AD are responsible only for less than 1 % of the total number of AD cases, the discovery of mutated genes, which trigger the development of the disease, is crucial for understanding the pathological processes. In particular this concerns research based on animal models.

The majority of AD cases occur in elderly (>65 years) and are commonly referred to as late-onset AD (LOAD). There are multiple environmental and genetic risk factors contributing to the developing of the LOAD (see 1.3.4), but impairment in Aβ clearance is probably a major contributor to disease pathogenesis (Liu et al. 2013).

1.3.3 Risk factors for Alzheimer's disease

The most significant risk factor for sporadic Alzheimer's disease is ***aging*** (Blennow et al. 2006). 12,5% people older than 65 years and 45% of the people older than 85 suffer from AD. Despite its high prevalence among older people, the disease is not considered to be a normal aging process (Alzheimer's Association, 2012).

Apart from aging, ***gender*** is an important risk factor for AD. The incidence of the disease is higher in women than in men (Alzheimer's Association, 2015), and this is not only due to higher life expectancy. Interestingly, mitochondria in young females are protected against amyloid-beta toxicity. They generate less reactive oxygen species and release less apoptogenic signals than mitochondria in males (Viña and Lloret 2010). However, this protective effect is lost in mitochondria in old post-menopausal females.

Thus, it is held that estrogenic compounds may protect against mitochondrial toxicity of amyloid-beta. This protective effect has been observed in a number of laboratories worldwide and suggests a method for treating or preventing AD by estrogenic compounds (Viña and Lloret 2010).

Epidemiological studies have suggested further risk factors for sporadic AD: vascular diseases like ***atherosclerosis***, ***hypercholesterinaemia***, ***coronary heart disease*** and ***heart failure*** (Kivipelto et al. 2001; Kivipelto et al. 2005; Qiu et al. 2006). In addition, several studies showed that ***obesity***, ***smoking*** and ***type II diabetes*** also increase the risk of developing AD (Kivipelto et al. 2005; Prince et al. 1994). Head injury and ***traumatic brain injuries*** are also regarded as risk factors for AD (McCullagh et al. 2001; Plassman et al. 2000; Sivanandam and Thakur 2012). Severe head injury leads to beta-amyloid protein deposition in the brain (Roberts et al. 1994), and increases a number of beta APP-immunoreactive neurons in the entorchial cortex (McKenzie et al. 1994).

Moreover, carrying at least one copy of the ***ApoE4*** allele increases the risk for sporadic AD (Corder et al. 1993). The Apolipoprotein E has a variety of functions. It is involved in microtubule stability, cell survival, cholesterol transport and amyloid deposition (Herz and Beffert 2000). The Apoprotein E gene is mapped to chromosome 19q. This gene is polymorphic and has three major alleles: ApoE2, ApoE3 and ApoE4. The presence of t ε4 allele is associated with increased risk for both early- and late-onset AD. Compared with individuals with an ε3/ε3 genotype, individuals with one copy of the ε4 allele developed AD much more frequently. Genome-wide association studies have verified that the ε4 allele of ApoE is the most significant genetic risk factor for sporadic AD (Liu et al. 2013). Furthermore, there are indications that Apolipoprotein E ε2 allele protect against late-onset AD (Corder et al. 1994).

Concearning protective factors, there are a variety of studies confirming that a healthy and physically active lifestyle together with cognitive stimulating activities and a Mediterranean diet reduce risk for Alzheimer's disease and protects against it (Fratiglioni et al. 2004; Gu et al. 2010; Hall et al. 2009) (see 1.8.2).

1.4 Diagnosis of Alzheimer's disease

Currently, a ***definite diagnosis*** of AD can only be confirmed by examining the brain *post mortem*. In 2011, the National Institute on Aging (NIA) provided guidance for documenting and reporting Alzheimer's-related brain changes observed during autopsy. According to this guideline, there are several characteristic lesions, which can be attributed to AD. For a neuropathological diagnosis of AD, neuropathological change has to be ranked along three essential parameters: ***Aβ plaques***, ***Neurofibrillary tangles (NFTs)*** and ***Neuritic plaques*** (Braak and Braak 1991; Hyman et al. 2012; McKhann et al. 1984).

Prior to the patient's death, cognitive and behaviour changes as well as the family history of a patient are used to diagnose possible AD (Alzheimer's Association, 2015).

In 1983, a group was convened by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) for the purpose of establishing ***criteria for the clinical diagnosis*** of AD. The report of the NINCDS-ADRDA work group (McKhann et al. 1984) contains issues of AD medical history, clinical examination, neuropsychological testing, and laboratory assessments. The criteria in this report (NINCDS-ADRDA criteria) have been quite successful during the quarter of the century. However, these criteria also required revision. Therefore, in 2011, the National Institute on Aging and the Alzheimer's Association convened a workgroup to revise the 1984 criteria for AD dementia. The NINDS–ADRDA criteria had to be reviewed and then updated reflecting modern innovations in clinical diagnosis, imaging, and laboratory assessment (McKhann et al. 2011). The National Institute on Aging and the Alzheimer's Association workgroup proposed the following terminology for classifying individuals with dementia caused by AD: *(1)* ***Probable AD dementia*** (dementia may be due to another cause), *(2)* ***Possible AD dementia*** (no other cause for dementia can be found), and *(3)* ***Probable or possible AD dementia with evi-***

dence of the AD pathophysiological process. *(1)* and *(2)* can be used in all clinical settings, *(3)* is intended for research purposes.

A detailed description of core clinical criteria for all-cause dementia, probable AD and possible AD is highlighted in *"The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging and the Alzheimer's Association workgroup"* (McKhann et al. 2011). In this chapter, I would like to mention only some exclusion criteria for AD. For example, the diagnosis of probable AD dementia ***should not*** be applied when there is evidence for substantial concurrent diseases of the following types (McKhann et al. 2011):

(1) cerebrovascular disease (a history of a stroke, which is temporally related to the onset of cognitive decline, or the presence of multiple or extensive infarcts or severe white matter hyperintensity burden);
(2) dementia with Lewy bodies;
(3) behavioural variant frontotemporal dementia;
(4) specified aphasia;
(5) other concominant active neurological disease;
(6) use of medication that could have a strong effect on cognition.

A variety of tests has been developed to diagnose AD. Among them, there are testing procedures such as the Mini-Mental Stage Examination (Folstein et al. 1975), the Clock-Drawing Test (Aprahamian et al. 2010; Sunderland et al. 1989) or the Cambridge Cognitive Examination (Schmand et al. 2000; Martinelli et al. 2014). For a probable diagnosis of AD, severe deficits that affect the patient in carrying out daily activities are essential (American Psychiatric Association 1995). Besides memory impairment, patients must show such symptoms as agnosia, aphasia, apraxia or impairments in executive functions (Waldemar et al. 2007). The diagnosis can be supported by neuroimaging (Magnetic Resonance Tomography, Computer Tomography, or Positron-Emission Tomography with radiolabeled glucose (FDG-PET[1]) or an amyloid-binding dye (PiB-PET[2]) (Ballard et al. 2011; Blennow et al. 2006; Perrin et al. 2009; Schroeter et al. 2009). With these methods, alternative causes of dementia like brain tumours, hydrocephalus or subdural hematomas can be excluded. In addition, analysis of biomarkers in the cerebrospinal fluid has been established for diagnosis of MCI and AD. Detection of reduced levels of $A\beta_{x\text{-}42}$ and increased levels of Tau and phosphorylated Tau can support a diagnosis (Fiandaca et al. 2014; Mattsson et al. 2009; Perrin et al. 2009).

An early diagnosis of AD is very important. It gives people opportunity to participate in clinical trials and research studies to test possible new treatments. If the disease is treated at the early stage, important capabilities can be preserved for some time. An early diagnosis also helps patients and their families to plan for the future.

[1] Fluordesoxyglucose positron emission tomography
[2] Pittsburgh compound B-positron emission tomography. PiB is a radioactive analog of thioflavin T.

1.5 Neuropathological hallmarks of Alzheimer's disease

1.5.1 Amyloid-beta deposition

Amyloid-beta deposition is one of the major neuropathological hallmarks of AD. Aβ depositions are specific formations composed of extracellular accumulation of the Amyloid-beta peptide (Aβ), which is derived from cleavage of Amyloid-Precursor-Protein (APP) (Holtzman et al. 2011; Serrano-Pozo et al. 2011). The particular Aβ species are classified with respect to their corresponding amino acid sequence e.g. $A\beta_{X1\text{-}X2}$ is an Aβ peptide, which starts from the N-terminal amino acid X1 and ends with the C-terminal amino acid X2.

The term ‘‘senile plaque’’ used for decades for description of Aβ accumulation is very unprecise. Duyckaerts et al. (2009) suggested in the review “Classification and basic pathology of Alzheimer disease” limit the use of this term. Duyckaerts et al. (2009) distinguish between mature Aβ deposition, ***neuritic plaque*** (especially when there is no referring to the technique that has been used to reveal the lesion), and other extracellular accumulation of Aβ, called ***deposits***. For clarity, speaking about Aβ deposits, it should be mentioned, how they have been revealed e.g. Aβ deposits can be revealed by anti-Aβ antibodies, and high density amyloid deposits can be revealed by Congo red or Thioflavin-S staining. A second possible qualifying term for deposits may be the description of their shape: diffuse, focal or stellate Aβ deposits (Delaère et al. 1991; Duyckaerts et al. 2009) (Figure 1.2).

In this thesis, I would like to distinguish between diffuse Aβ deposits, calling them ***diffuse plaques,*** and ***neuritic plaques.***

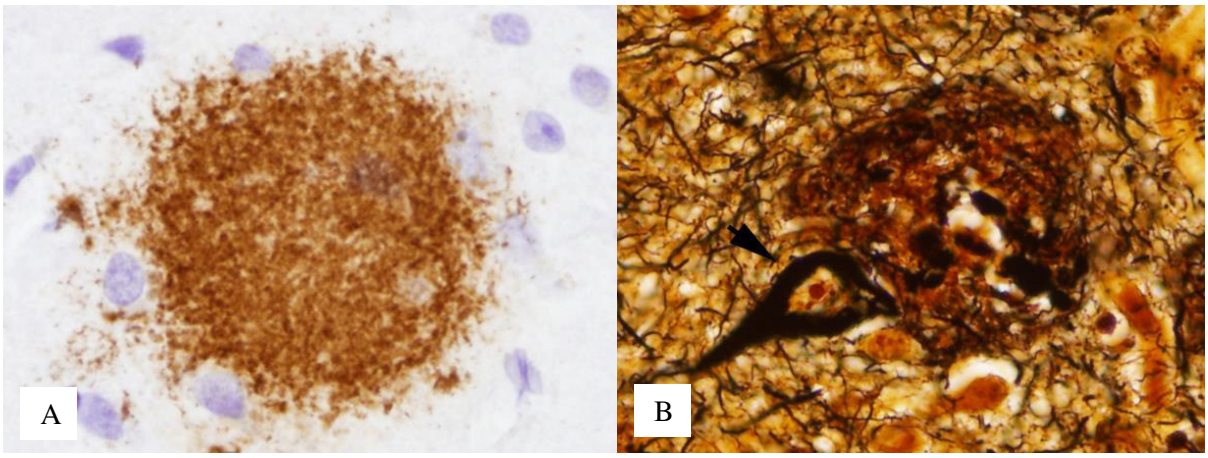

Figure 1. 1: Two types of amyloid plaques. (A) Diffuse plaques (brownish cloud). Aβ immunohistochemistry. (B) Neuritic plaque (big dark clew in the middle of the image) with neurofibrillary tangle (arrow). Neuritic plaques can contain reactive astrocytes and microglia, and are surrounded by swollen, degenerating axons and dendrites. Figures reproduced from www.neuropathology-web.org

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Diffuse plaques (Figure 1.1A) are composed of non-fibrillary depositions of Aβ with almost no detectable neuritic dystrophy. They can be revealed using Aβ immunohistochemistry. Their diameter can vary from 50 µm to several hundred micrometres (Duyckaerts et al. 2009; Yamaguchi et al. 1988). Diffuse plaques are also found in healthy older individuals. This led to the hypothesis that an increase of the plaque load is associated with preclinical AD (Dickson et al. 1992; Knopman et al. 2003; Vlassenko et al. 2011).

Neuritic plaques (Figure 1.1B) are extracellular Aβ plaques which consist of highly aggregated fibrillary Aβ. These plaques are surrounded by swollen, degenerating axons and dendrites (Nelson et al. 2009). Plaque density and size can differ (with diameter from 10 to 120 µm) (Thomas and Fenech 2007). Neuritic plaques can be stained with β-sheet staining dyes such as Thioflavin-S and Congo red indicating their fibrillary structure (Serrano-Pozo et al. 2011). In the immediate vicinity to neuritic plaques, a variety of neuropathological changes is observed: neuritic dystrophies, degenerated neurons, as well as astro- and microgliosis (see 1.5.3) (Holtzman et al. 2011; Lenders et al. 1989; Masliah et al. 1990; Pike et al. 1995b; Selkoe 2011; Urbanc et al. 2002). Besides Aβ, other proteins such as APP, Tau and Ubiquitin are found in neuritic plaques (Su et al. 1998; Duyckaerts et al. 2009).

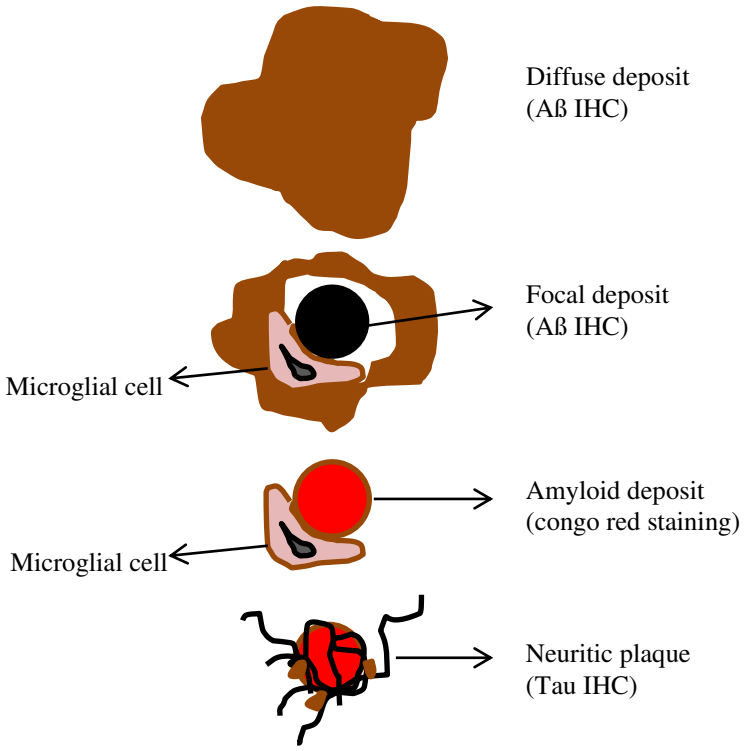

Figure 1. 2: Schematic overview over terms used to describe amyloid depositions. Central aspects of Aβ deposition revealed by different techniques are illustrated. The terms applied to qualify the focal deposits depend on the technique used to reveal them, for instance Aβ- and Tau-immunohistochemistry (IHC), Congo red or Thioflavin-S. Tau-immunohistochemistry only reveals neuritic plaques. The vertical position of the figures should represent the sequence of events leading to the neuritic plaque. Stellate deposits are not shown. Figure created after Duyckaerts et al. 2009, p. 7.

The amyloid deposition typically starts in the neocortex, and then affects the hippocampus, basal ganglia and cerebellum (Serrano-Pozo et al. 2011). In the final stage of AD,

neuritic plaques are also found in subcortical structures such as the brainstem (Aldwin and Gilmer 2013; Arnold et al. 1991; Thal et al. 2002).

In spite of the fact that plaques are a key feature in the diagnostic of AD, the absolute plaque load correlates poorly with cognitive impairment and disease stage (Arriagada et al. 1992; Billings et al. 2005; Giannakopoulos et al. 2003; Schaeffer et al. 2011; Villemagne et al. 2011). Approximately 80 % of the AD patients also show the symptom of amyloid deposition in blood vessels, called Cerebral Amyloid Angiopathy (CAA) (Glenner and Wong 1984).

1.5.2 Neurofibrillary tangles

Neurofibrillary tangles (NFT) are intracellular formations consisting of hyperphosphorylated Tau protein organized in paired helical filaments (Kidd 1963; Grundke-Iqbal et al. 1986; Lee et al. 1991) (Figure 1.3). Tau is a phosphoprotein expressed in all nucleated cells and is abundant in neurons (Duyckaerts et al. 2009; Galimberti and Scarpini 2012). Already Alois Alzheimer mentioned NFTs in his report as “intraneuronal filamentous inclusions” (Alzheimer 1907). Tau is a microtubule-associated protein and considered to be a significant factor in the axonal growth, development of neuronal polarity, and the maintenance of microtubule dynamics (Higuchi et al. 2002). In its normal function, Tau is involved in the organization of cytoskeleton (Drechsel et al. 1992; Gustke et al. 1994; Weingarten et al. 1975; Witman et al. 1976), whereby it binds to tubulin assembling and stabilizing microtubules (Goedert and Spillantini 2006). These functions of tau are primarily regulated by the activities of protein kinases and phosphatases (Higuchi et al. 2002). Hyperphosphorylation of Tau reduces tubulin’s binding activity and results in a higher disposition to form paired helical filaments (Alonso et al. 1996; Holtzman et al. 2011). This leads to the breakdown of the microtubule network and consequently to neurofibrillary degeneration (Iqbal and Grundke-Iqbal 2008).

Tau is essential for diagnosing and staging of AD (Arnold et al. 1991; Braak and Braak 1991). During the course of AD, Tau aggregation and NFT formation appear later than amyloid deposition (Galimberti and Scarpini 2012). In contrast to amyloid deposition, the NFT formation correlates with progression of the cognitive deficits (Braak and Braak 1991; Holtzman et al. 2011). In some cases the formation of fibrillary bundles in the entorhinal cortex starts many years before the first symptoms of dementia appear. Subsequently, they spread to the hippocampus and to the neo-cortex (Braak and Braak, 1991). It has been shown that the regional number of NFTs highly correlates with the loss of synapses and the stage of cognitive impairment in AD patients (Masliah et al. 1992).

Moreover, there is a variety of so called “tauopathies” – diseases that characterised by neurodegeneration directly induced by pathological aggregates of tau in different filamentous structures such as paired helical filaments, straight filaments, and neuropil threads in the axons and dendrites. For example, familial missense mutations in the Tau gene cause another form of dementia called frontotemporal dementia with parkinsonism (FTDP-17) (Hutton et al. 1998; Spillantini et al. 1998). Pick’s Disease (Probst et al. 1996), cortico-

basal degeneration (Matsumoto et al. 1996), and dementia pugilistica (Hof et al. 1992) also belong to the group of "tauopathies".

However, in the case of AD, there is evidence from *in vitro* and *in vivo* models showing that accumulation of amyloid beta peptides is a starting point of the pathology, and deposition of Aβ consequently initiates the fibrillization of tau (Guo et al. 2006).

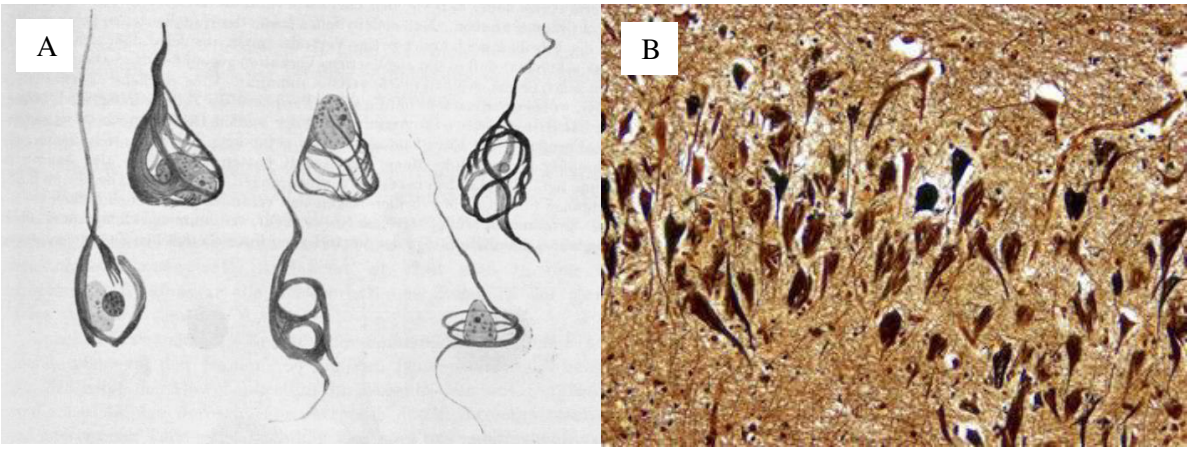

Figure 1. 3: Neurofibrillary tangles. (A) A diagram of the tangles from a 1911 research paper by Alois Alzheimer (Alzheimer 1911). Figure reproduced from http://nyamcenterforhistory.org/. (B) Image of neurofibrillary tangles in cerebral cortex of a patient with Alzheimer's disease visualized with Bielschowsky silver stain. Figure reproduced from www.neuropathology-web.org

1.5.3 Inflammation

The role of inflammation in AD brain is not fully understood. Neuroinflammation is consistently found in AD patients (Hoozemans et al. 2006) and also occurs in transgenic models of AD (Benzing et al. 1999; Matsuoka et al. 2001; Dudal et al. 2004). Activated astrocytes and microglia, the complement system as well as cytokines and chemokines are found in the proximity of neuritic plaques. This leads to the conclusion that Aβ triggers an inflammation process via glia activation (Itagaki et al. 1989; Krause and Müller 2010; Pike et al. 1995b). Following activation, microglia and astrocytes release proinflammatory signal molecules including complement factors, chemokins and cytokines (Rubio-Perez and Morillas-Ruiz 2012; Tuppo and Arias 2005). Several studies suggest that glial cells take up and process Aβ (Koenigsknecht-Talboo et al. 2008; Meyer-Luehmann et al. 2008). However, it remains still unclear whether inflammatory processes are generally harmful and induce neurodegeneration (Hauss-Wegrzyniak et al. 1998). This is just as plausible as understanding them as a protective reaction in an attempt to rescue neurons (Weninger and Yankner 2001). It was suggested that glial cells contribute to the elimination of toxic Aβ species (Aβ clearance), thus, it might be profitable for the treatment of AD (Bard et al. 2000; DeMattos et al. 2012).

1.5.4 Brain atrophy and neuron loss

Atrophic changes and neuron loss are one of the major hallmarks of AD brain, still they have been also found in other dementias such as frontotemporal dementia or vascular dementia (Blennow et al. 2006). The main structures that undergo massive neuronal loss are the entorhinal cortex, the hippocampus (Mizutani et al. 1990), the posterior cingulate cortex, the amygdala, and the temporo-parieto-occipital association cortices (Brun and Englund 1981) but not the inferior frontal and orbitofrontal gyri (Blennow et al. 2006; Duyckaerts et al. 2009; Halliday et al. 2003). These anatomical observations correlate with the psychiatric disorders of patients suffering from AD. In addition, a strong correlation of neuron number and hippocampal/brain volume has been reported (Kril et al. 2004). Furthermore, it has been shown that magnetic-resonance brain imaging can be used to trace the progression from mild cognitive impairment to AD by estimation of hippocampal/brain atrophy (Jack et al. 2005; Jagust 2006). Brain atrophy mainly caused by neuron loss is the most evident macroscopic characteristic of AD (Figure 1.4).

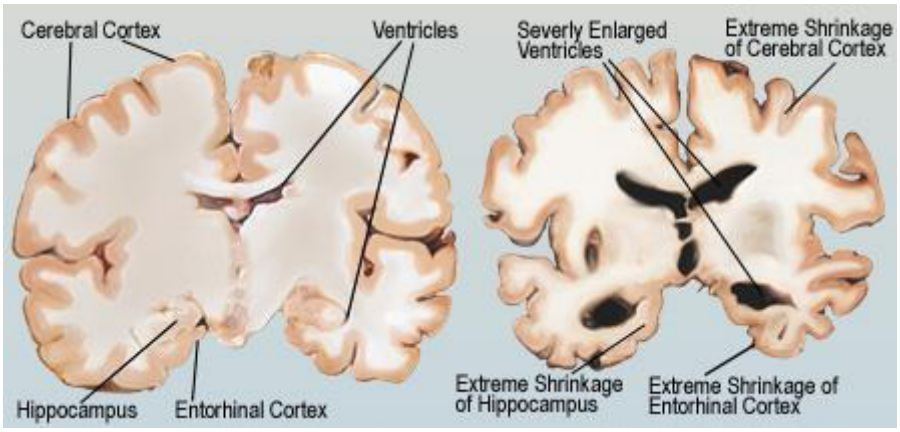

Figure 1. 4: Sagittal sections of brains. Normal adult brain (on the left side) compared with Alzheimer brain (on the right side) showing marked diffuse atrophy in the cortical regions and ventricular enlargement. Figure reproduced from http://memory.ucsf.edu/education/diseases/alzheimer

However, the cause of brain atrophy/neuronal loss remains a subject of ongoing dispute. Most recent reports suggest that intracellular accumulation of aggregated Aβ is crucial for cell death and neuron loss (Bayer and Wirths 2010; Haass and Selkoe 2007; Larson and Lesné 2012). Others reported a correlation of brain region atrophy with the number of NFTs within the same brain region (Cras et al. 1995; Gómez-Isla et al. 1997).

Design based stereology showed a significant neuron loss in the entorhinal cortex of patients with very mild AD (Gómez-Isla et al. 1996). More than 50% of the neurons in the superior temporal sulcus are lost in patients with AD (Gomez-Ila et al. 1997). A massive neuron loss in the CA1 was reported in AD patients (West et al. 2004), whereas no neuron loss was observed in preclinical AD. Furthermore, brain neuronal loss is observed in various murine models of Alzheimer's disease (Bouter et al. 2014; Casas et al. 2004; Christen-

sen et al. 2008; Christensen et al. 2010; Jawhar et al. 2012; Meißner et al. 2015; Oakley et al. 2006; Saul et al. 2013; Schmitz et al. 2004; Wirths and Bayer 2010).

Synapse loss is also an early sign of the pathological processes in AD. It has been noticed that the number of lost synapses surpasses the neuron loss in the cortex. It allows assuming that synapse loss starts before neuron loss and contributes to the cortical atrophy (Serrano-Pozo et al. 2011). In addition, decreased synaptic density correlates directly with the cognitive decline in AD patients. According to some studies, synaptic density is considered to be a better correlate of severity of AD compared to NFTs or neuron loss (DeKosky and Scheff 1990; Ingelsson et al. 2004; Scheff and Price 1993; Scheff et al. 1990).

1.5.5 Alterations in neurogenesis

Even the adult brain continually generates new neurons. Animal studies showed that the synthetic nucleoside bromodeoxyuridin (BrdU) was incorporated in cells of the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the mammalian brain (Kaplan and Hinds 1977). These cells were confirmed to be new-born neurons giving evidence about existence of a mitotic activity in those regions.

Newly generated cells from the SVZ migrate to the olfactory bulb where they differentiate into functional neurons, while new-born cells issued from the SGZ of the dentate gyrus migrate and become integrated in the granule cell layer of the dentate gyrus (Cayre et al. 2009; Crews and Nixon 2003) (Figure 1.5).

In the context of Alzheimer's disease, adult hippocampal neurogenesis attracts huge attention, because it is involved in higher cognitive function, especially memory processes, and certain affective behaviours. Adult hippocampal neurogenesis appears to contribute significantly to hippocampal plasticity during lifetime, because it is regulated by activity. New excitatory granule cells are generated in the dentate gyrus, whose axons form the mossy fibre tract that connects the dentate gyrus to the CA3 region. Neurogenesis starts from a population of radial glia-like precursor cells (type 1 cells) that have astrocytic properties. They express markers of neural stem cells and divide rarely. Type 1 cells give rise to intermediate progenitor cells with first glial (type 2a) and then neuronal (type 2b) phenotype. Going through a migratory neuroblast-like stage (type 3), the new-born cells exit the cell cycle and enter a maturation stage. During maturation stage, they extend their dendrites into the molecular layer and their axon to the CA3 region. During a period of several weeks, the new cells show increased synaptic plasticity, before finally being integrated and becoming indistinguishable from the older granule cells (Kempermann et al. 2015) (Figure 1.6).

It was discovered that endogenous and environmental factors, as well as drugs can modulate the neurogenesis in a positive or negative way (see also 1.8.2). For example, physical activity (van Praag et al. 1999), as well as hormonal levels (Cameron and Gould 1994), diet (Stangl and Thuret 2009), or cannabinoids (Jiang et al. 2005) and antidepressant drugs (Boldrini et al. 2009) can affect the number of new-born neurons (Figure 1.7).

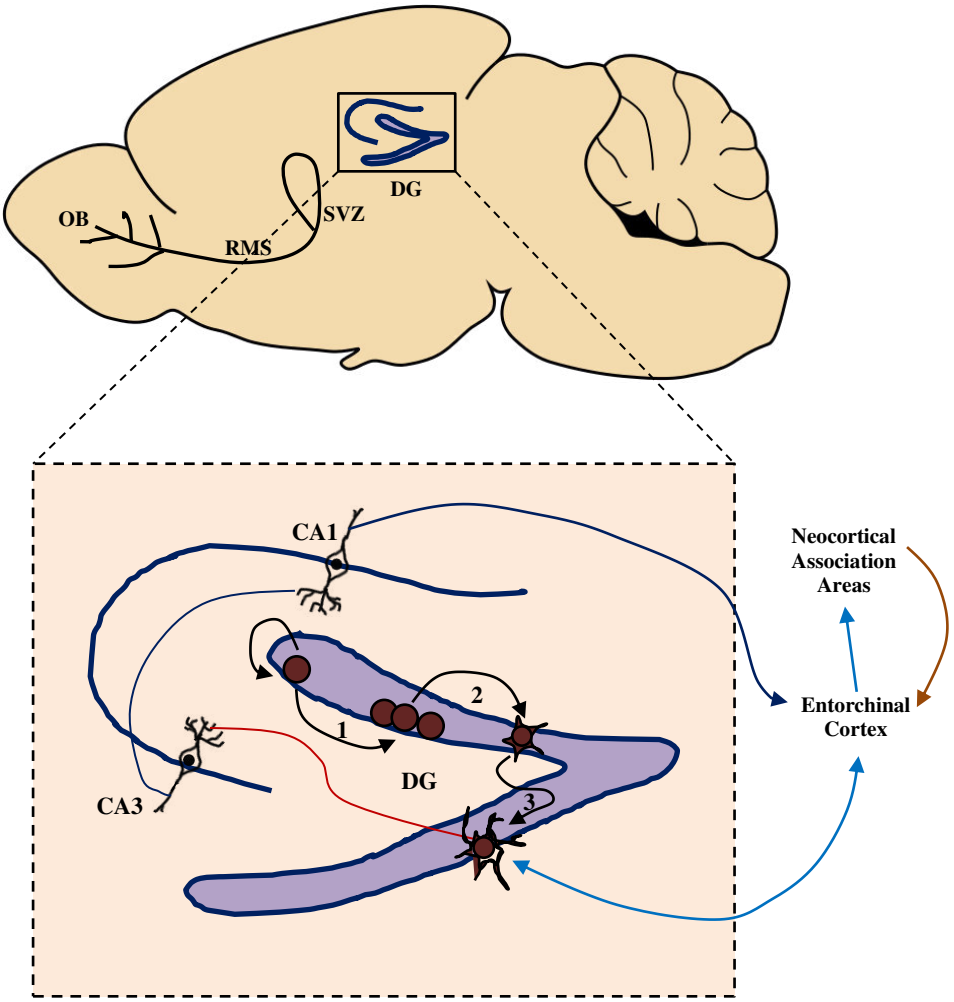

Figure 1. 5: Schematic sagittal view of a rodent brain pointing out the two neurogenic zones of the adult mammalian brain: the subventricular zone (SVZ) of the anterior lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. New-born neurons generated in the SVZ of the anterior lateral ventricles migrate through the rostral migratory stream toward the olfactory bulb where they become functional neurons. Cells dividing in the SGZ of the dentate gyrus become incorporated into the granular cell layer before completely differentiate into granule cells. The enlarged hippocampal region (black square) shows (1) neural progenitor cells in the SGZ of the DG proliferating, (2) migrating into the granule cell layer and (3) maturing into new granule neurons. These integrate into the hippocampal circuitry by receiving inputs from the entorhinal cortex and extend projections into the CA3. Figure created after Stangl and Thuret 2009, p. 273.

However, the role of neurogenesis in memory and learning is still not well understood as a wide range of studies show contradictory results. It was shown that blocking hippocampal neurogenesis results in better performances in hippocampus-dependent memory tasks (Saxe et al. 2007). Other studies showed the opposite effect: the inhibition of neurogenesis decreased learning abilities (Hu et al. 2009; Jessberger et al. 2009). Neurogenesis is also a subject of discussion in the field of Alzheimer research due to controversial findings about the neurogenic behaviour of diseased brains. Indeed, neurogenesis was found to be either increased or decreased both in rodents and humans (Chevallier et al. 2005; Haughey et al. 2002; Taupin 2009; Ziabreva et al. 2006).

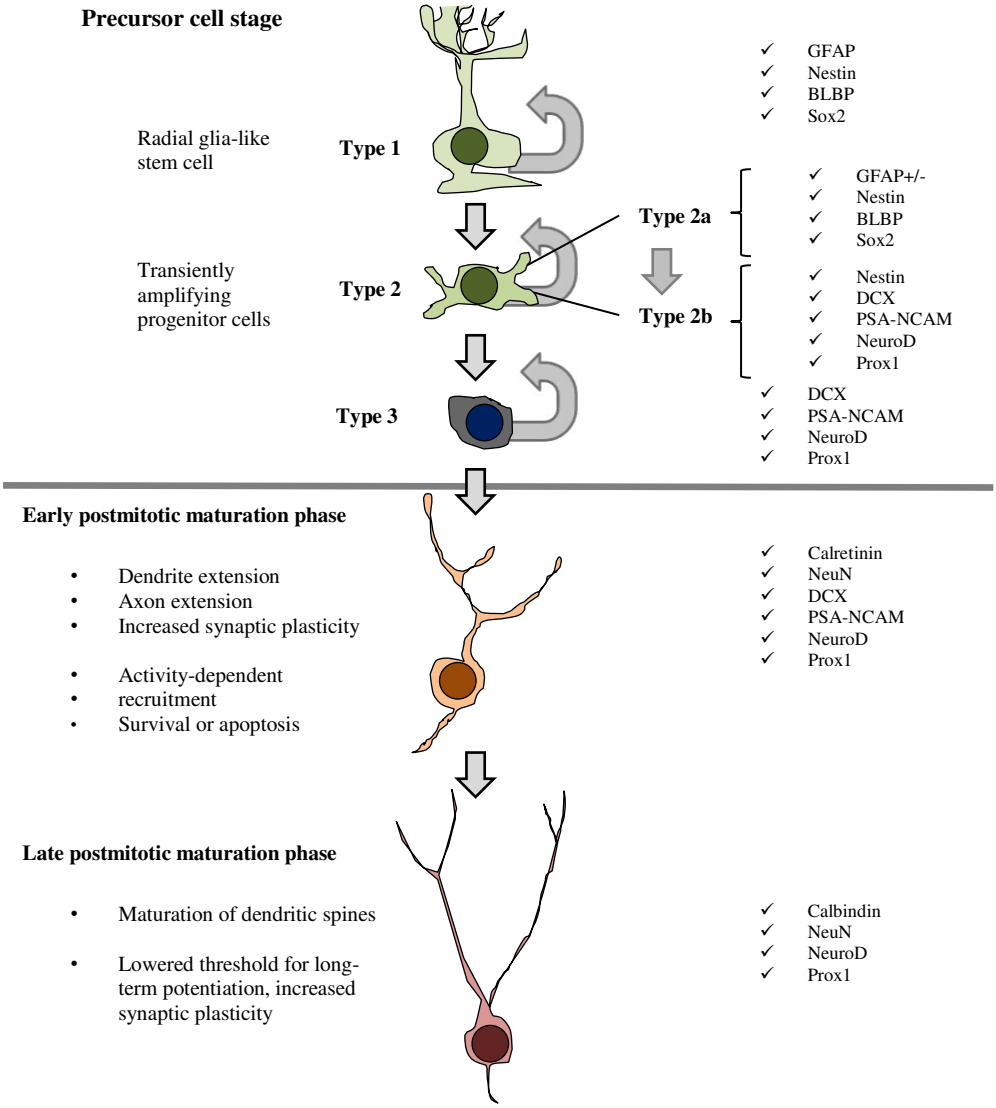

Figure 1. 6: Developmental stages in the course of adult hippocampal neurogenesis (GFAP, Glial fibrillary acidic protein; BLBP, brain lipid-binding protein; DCX, doublecortin; PSA-NCAM, polysialilated neural-cell-adhesion molecule). Figure created after Kempermann et al. 2015, p. 4.

Further facts about adult neurogenesis under other pathological conditions are very interesting as well. Following brain trauma or ischaemia, proliferation is increased in the dentate gyrus and the SVZ (Yagita et al. 2002; Kokaia and Lindvall 2003; Parent 2003). In addition, new-born neurons can also be found in the striatum and neocortex. These latter effects have been ascribed to the atypical migration of SVZ neuronal precursors towards damaged areas. Therefore, pathological conditions might induce endogenous neurogenesis in regions where adult neurogenesis is usually non-existent (Dash et al. 2001; Zhang et al. 2004).

Despite controversial discussion, adult neurogenesis is considered to be a serious therapeutic target in AD treatment, because of the multiple possibilities of its modulation. This topic will be discussed in section 1.8.1.

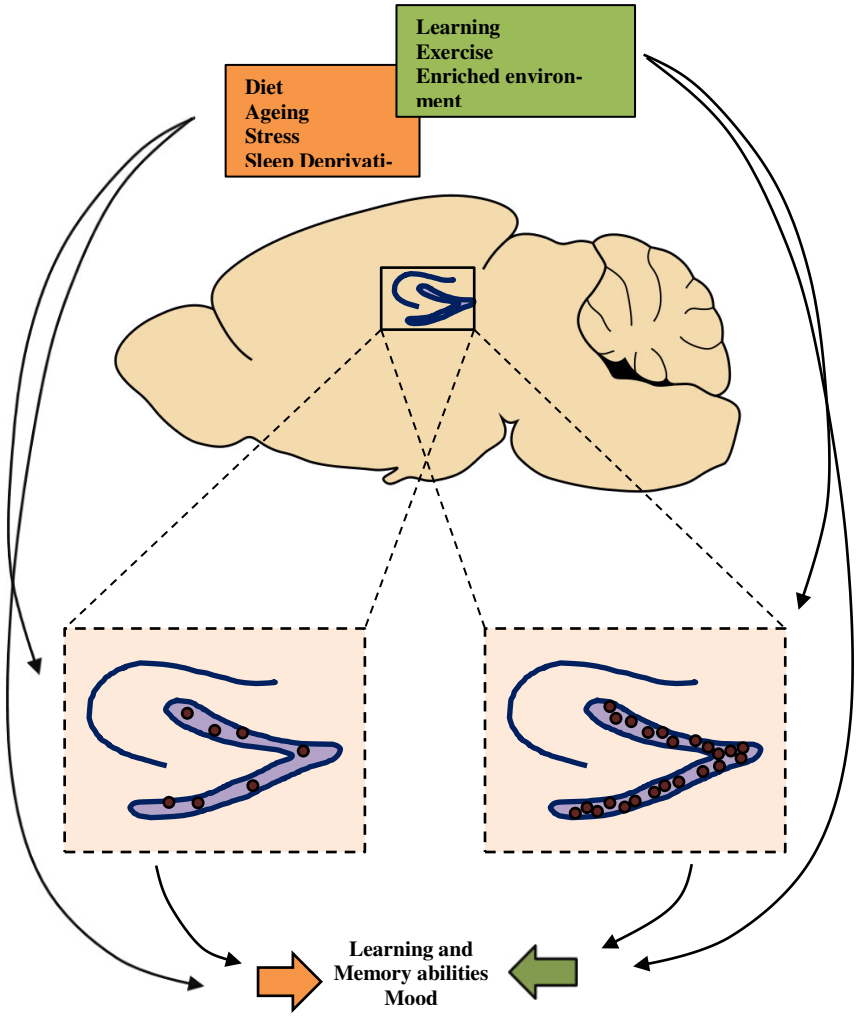

Figure 1. 7: Overview of the physiological and environmental modulation of adult hippocampal neurogenesis and its influence on learning, memory abilities, and mood. In the enlarged hippocampus (dotted squares), the red dots symbolize newborn neurons in the dentate gyrus (DG)**.** Figure created after Strangl and Thuret 2009, p. 276.

1.5.6 Motor impairment. Axonopathy

One of the most relevant symptoms at the later stages of AD is the progressive decline of motor functions.

Along with cognitive decline, motor deficits make patients helpless and dependent even in performing the simplest motor tasks, and burden their caregivers (Flannery 2002). Motor deficits can be so severe that patients are not able to swallow properly and can choke with meal. That can lead to aspiration pneumonia and death (Wada et al. 2001). At the early stages of AD, this symptom manifests mainly in early deficits in balance (Rolland et al. 2009) and motor coordination (Persad et al. 2008; Yan et al. 2008) increasing the risk of falls (Allan et al. 2009). Decline in motor performances is regarded as a potential predictor of AD progression and severity (Hebert et al. 2008) and can help distinguishing between mild cognitive impairment and early stages of AD (Pettersson et al. 2005). Paying attention to the early motor impairments may enable earlier pharmacological or environ-

mental interventions, which could slow down the course of AD. A range of transgenic mouse models of AD also showed age-dependant motor impairments (Jawhar et al. 2012; Wirths and Bayer 2008; Wirths et al. 2010b). It has been hypothesised that motor deficits can be explained by insufficient cerebral blood flow in the visual area (Nakano et al. 1999) or hyperexcitability of neuronal networks of the motor cortex (Ferreri et al. 2003).

It was also noticed that prominent defects in axonal transport correlate with impairment of motor performance and degenerative processes (Chevalier-Larsen and Holzbaur 2006; Wirths et al. 2007). Thus, ***impaired axonal transport*** also may be the cause of motor deficits.

Axonal defects can manifest itself as axonal swellings or spheroids, which correspond to axonal enlargements and aberrant accumulation of axonal cargoes, cytoskeletal proteins and lipids (Wirths et al. 2007) (Figure 1.8). Swollen axons and dendrites with these accumulations are often called dystrophic neurites, and the range of all axonal abnormalities and deficits is called axonopathy.

The observation of abnormal filaments within the cytoskeleton of dystrophic neurites in *pre* and *post mortem* brain tissue of AD patients led to the hypothesis that dysfunctional fast axonal transport could be part of the AD pathology (Praprotnik et al. 1996). Cortical neurons extracted *post mortem* from temporal cortex of AD patients showed significantly impaired axonal transport in comparison to healthy controls (Dai et al. 2002). Further animal studies showed for instance that in vivo application of $A\beta_{1-42}$ blocks fast axonal transport in rat sciatic nerve (Kasa et al. 2000).

The production of neurotoxic Aβ and the formation of hyperphosphorylated Tau are supposed to be crucial steps contributing to the neuropathological mechanisms in Alzheimer's disease (AD). However, the exact relationship between axonopathy and the origin and development of classic neuropathological changes such as amyloid plaques and NFTs remains unclear (Xiao et al. 2011).

Axonal deficits and impairment of motor functions are common pathological alterations in mouse models expressing different isoforms of human mutant tau protein (Wirths and Bayer 2008).

However, in recent years, similar phenotypes and indications for disorders in axonal transport and axonopathy have been also described in different APP-based transgenic AD mouse models (Adalbert et al. 2009; Jawhar et al. 2012; Salehi et al. 2006; Stokin et al. 2005; Wirths et al. 2006; Wirths et al. 2007) and in APP-based models of Down syndrome (Salehi et al. 2003).

One of the suggested mechanisms of fast axonal transport disruption in AD implied that mutant presenilins induce phosphorylation of the kinesin light chain by GSK3β that disrupts the attachment of kinesin to vesicles. Therefore, the vesicles are not being transported. GSK3β also plays a role in the hyperphosphorylation of Tau. In its hyperphosphorylated form, Tau disorganizes the microtubule structure and interrupts the traffic of vesicles (De Vos et al. 2008). A block of axonal transport can also cause "dying back" axon degeneration (Coleman 2005; Ferri et al. 2003) and cell death (Hafezparast et al. 2003; LaMonte et al. 2002).

Moreover, a scientific debate has raged on the issue whether Aβ acts as a trigger for impaired axonal transport in the pathophysiology of AD (Christensen et al. 2014; Wirths et

al. 2007). Evidence that axonopathy happens *in vivo* without Tau hyperphosphorylation (Wirths et al. 2007) indicates that intraneuronal Aβ can also be a potent factor for disrupting axonal transport (Bayer and Wirths 2008; Pigino et al. 2009). However, it is still unclear, whether axons become so severely dystrophic because of their proximity to amyloid plaques, or whether amyloid plaques, which are situated closely to the damaged axons, are partly composed from proteins leaking from the dystrophic neurites. Direct leakage of intra-axonal components, including structural and functional proteins, to extra-axonal space may be involved in the formation of plaques. For instance, the leakage of overproduced intra-axonal Aβ or extra-axonal proteolysis of the leaked APP may result in Aβ plaques (Xiao et al. 2011).

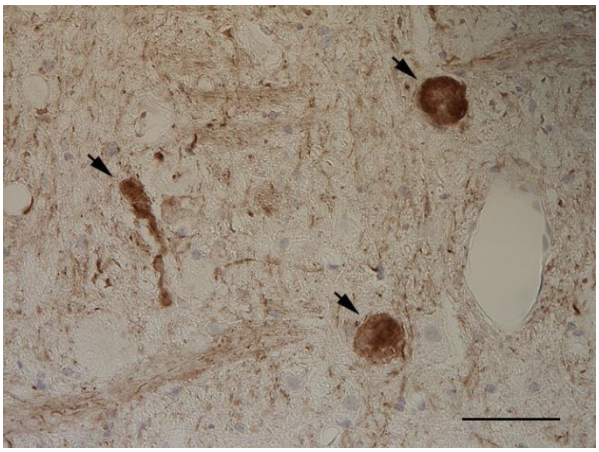

Figure 1. 8: Axonal swellings. Neurofilament-L stained brain section of 5XFAD mouse showing axonopathy (arrows = swollen axons); scale bar: 33 µm. Source: own research.

1.6 Pathomechanisms of Alzheimer's disease

1.6.1 The Amyloid Precursor Protein

The amyloid precursor protein (APP) is a type-1 transmembrane glycoprotein, which is highly conserved and is ubiquitously expressed. APP belongs to the amyloid-precursor-like protein family (Puzzo et al. 2014; Selkoe et al. 1988). APP as transmembrane glycoprotein consists of three parts: the intracellular region, the extracellular region, which is much larger than the intracellular one, and the transmembrane domain (Castellani et al. 2009; Kandel et al. 2000).

In humans, the APP gene is located on chromosome 21. Aβ peptides, which are the main components of plaques and which are responsible for AD and other amyloidosis, are derived from sequential cleavage of APP (Castellani et al. 2009; Korenberg et al. 1989).

Though the members of the amyloid-precursor-like protein family are structurally highly conserved, they show large heterogeneity in the Aβ region (Selkoe 2001). Through alternative splicing and different post-translational modifications, a variety of isoforms of

APP are expressed in different types of tissues. These isoforms are categorised by the number of amino acid they contain. Among them, APP_{695}, APP_{751} and APP_{770} are the three major forms expressed in neuronal cells (Sandbrink et al. 1996). APP_{695}, is mainly expressed in neurons of the central nervous system. The highest levels of its expression have been detected in the cortex, hippocampus and cerebellum (Solà et al. 1993). APP_{751} and APP_{770} are also widely expressed in other tissues (O'Brien and Wong 2011; Selkoe 2001).

Several physiological functions have been attributed to APP: synaptogenesis, neurite outgrowth, protein transport along the axons, cell adhesion and calcium metabolism (Zheng and Koo 2006). APP is also thought to be involved in neuronal migration (Young-Pearse et al. 2007) and gene transcription involved in cell death (Kögel et al. 2005). In spite of this, the role of APP still remains not well understood. For instance, APP knockout mice are not affected in their vital functions and bear viable offspring. However, APP knockout mice show cerebral gliosis, defects in learning and long term potentiation, and reduced body and brain size (Dawson et al. 1999; Ring et al. 2007; Zheng et al. 1995). It has been suggested that the homologous APP-like proteins may partly perform the functions of APP in knockout mice so that the lethal phenotype can be avoided (Heber et al. 2000; Slunt et al. 1994).

1.6.2 APP processing

1.6.2.1 Non-amyloidogenic pathway

APP can be processed via two alternative pathways: the non-amyloidogenic and the amyloidogenic pathway (De-Paula et al. 2012) (Figure 1.9). In ***the non-amyloidogenic pathway***, APP is cleaved within the Aβ region by an ***α-secretase*** releasing the sAPPα (a large soluble N-terminal fragment) and a smaller C-terminal fragment C83 (Esch et al. 1990; Sisodia et al. 1990), which is further cleaved by ***γ-secretase*** releasing the soluble C-terminal p3 fragment and the APP intracellular domain (AICD) (Carrillo-Mora et al. 2014; Querfurth and LaFerla 2010). Various enzymes have been suggested to act as α-secretase. Among them are three members from the ADAM family (a disintigrin and metalloprotease family): $ADAM_9$, $ADAM_{10}$ and $ADAM_{17}$ (Haass 2004; Haass et al. 2012). Recent studies indicate that $ADAM_{10}$ functions as the main α-secretase in neurons (Kuhn et al. 2010).

After cleavage of C83 by γ-secretase, the p3 fragment rapidly degenerates (De-Paula et al. 2012), while AICD is supposed to act as transcriptional regulator (Chow et al. 2010). Because the cleavage of APP by α-secretase occurs within the Aβ region, the generation of amyloid peptides is prevented (Sisodia 1992). In contrast to Aβ species, sAPPα has been suggested to have neuroprotective activity (Chow et al. 2010; Furukawa et al. 1996; Mattson 1997).

1.6.2.2 Amyloidogenic pathway

The amyloidogenic pathway (Figure 1.9) is a mechanism of APP cleavage, which results in release of Aβ (De-Paula et al. 2012). In contrast to the non-amyloidogenic pathway, here APP is first cleaved by a β-secretase releasing a soluble N-terminal fragment (sAPPβ) and a longer C-terminal fragment C99. β-secretase cleaves the amyloid precursor protein 16 amino acids upwards from the α-secretase cleavage site. The membrane-bound C99 fragment is subsequently processed by γ-secretase. γ-secretase cleaves C99 within the transmembrane domain at different sites producing Aβ peptides of various lengths and AICD. The length of Aβ peptides range from 37 to 43 aminoacids (Annaert and Strooper 2002; Citron et al. 1995; De-Paula et al. 2012; Vassar et al. 1999; Wolfe 2007; Zhang et al. 2011). Physiologically, Aβ mainly consists of 40 amino acids. Under normal physiological conditions, the 42 amino-acid long Aβ, which constitutes amyloid plaques, is only a minor portion of the total Aβ (Zhang et al. 2011).

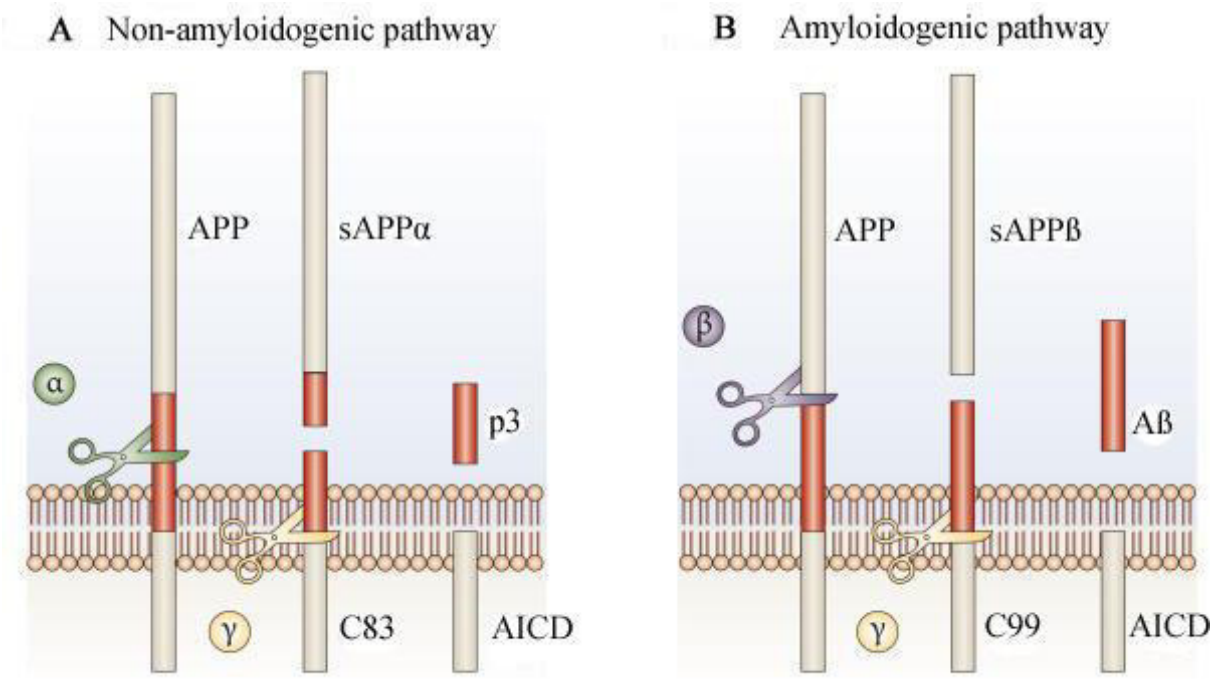

Figure 1. 9: APP processing by secretases. Schematic diagram showing non-amyloidogenic (A) and amyloidogenic pathways (B). (A) In the non-amyloidogenic processing, APP is first cleaved by α-secretase within the Aβ sequence releasing sAPPα. Then, the resulting carboxy-terminal fragment is processed by γ-secretase releasing the p3 fragment and the APP intracellular domain (AICD). No Aβ is produced. (B) The amyloidogenic pathway is initiated when β-secretase (BACE1) cleaves APP at the amino terminus of the Aβ peptide and releases sAPPβ. Further cleavage of the resulting carboxyl terminal by γ-secretase results in the release of Aβ and AICD. Abbreviations: Aβ, amyloid-β; APP, amyloid precursor protein; sAPPα, soluble amyloid precursor protein-α; sAPPβ, soluble amyloid precursor protein-β; AICD, APP intracellular domain; C83, carboxy-terminal fragment 83; C99, carboxy-terminal fragment 99. Figure modified from De Strooper et al. 2010, p. 101.

Various enzymes are acting as ***β-secretase***. The β-site cleaving enzyme 1 (BACE1) was identified as the primary β-secretase (Hussain et al. 1999; Sinha et al. 1999; Vassar et

al. 1999; Yan et al. 1999). BACE1 is a membrane-bound aspartyl protease and can cleave Aβ at two known positions: the aspartate at position 1 and the glutamate at position 11 (Sinha et al. 1999; Vassar et al. 1999). BACE1 is highly expressed in the brain, particularly in regions affected by AD (Yang et al. 2003; Vassar et al. 1999; Johnston et al. 2005). BACE2 and cathepsin B have been suggested as additional β-secretases (Hook et al. 2005; Hook et al. 2009). The physiological contribution of these enzymes to APP processing requires further investigation

The ***γ-secretase*** involved in the amyloidogenic pathway, is a complex protease composed of several components: the membrane-bound proteins presenilin 1 or presenilin 2, nicastrin, anterior pharynx defective-1, presenilin enhancer protein-2, and the cluster of differentiation 147 (Kaether et al. 2006; O'Brien and Wong 2011; Zhou et al. 2006).

Several studies have indicated that upregulation of α-secretase activity leads to a lower production of Aβ in subcellular compartments (Nitsch et al. 1992; Postina et al. 2004).

1.6.3 The amyloid cascade hypothesis

1.6.3.1 The classic amyloid cascade hypothesis

In the early 90s, the amyloid cascade hypothesis has been proposed by Hardy and Higgins (1992) to explain the etiology and pathogenesis of Alzheimer's disease (Hardy and Higgins 1992). According to this hypothesis, the deposition of Aβ peptides is the cause of all the neurodegenerative processes found in SAD and FAD such as neurofibrillary tangles, synaptic deficits, neuron loss, inflammation, vascular damage, and cognitive decline (Duyckaerts et al. 2009; Hardy and Allsop 1991; Selkoe 1991) (Figure 1.10). The hypothesis states that increased Aβ production or decreased Aβ clearance result in the accumulation of hydrophobic $A\beta_{40}$ and $A\beta_{42}$ and subsequent formation of insoluble extracellular Aβ plaques. Further, Aβ plaques trigger a cascade of changes resulting in dementia and all of neuropathological alterations mentioned above (Hardy and Allsop 1991; Pimplikar 2009).

The amyloid cascade hypothesis has been supported by the following findings:

(1) Numerous familial forms of AD were characterized, in which the inherited mutations in APP, PSEN1 and PSEN2 alter the Aβ levels with a significant increase in the $A\beta_{1\text{-}42}/A\beta_{1\text{-}}40$ ratio and invariably lead to increased depositions of Aβ in plaques (Bertram et al. 2010; Murayama et al. 1999; Pimplikar 2009; Rossor et al. 1993).

(2) Many AD mouse models with familial AD mutations also develop similar age-dependant pathologies such as Aβ plaques, gliosis and cognitive deficits (Duyckaerts et al. 2008; Pimplikar 2009).

(3) Down syndrome patients, which are carriers of an additional chromosome 21 and consequently an additional copy of the APP gene, also develop AD pathology with amyloid plaques and NFTs (Rumble et al. 1989; Schupf and Sergievsky 2002).

(4) ε4 allele of the Apolipoprotein E gene, which are considered to be the most important risk factor for late-onset AD, is associated with increased Aβ deposition and reduced Aβ clearance (see 1.3.3) (Bickeböller et al. 1997; Castellano et al. 2011).

Several other observations strengthened the theory of Hardy and Higgins. For example, mutations in the Tau gene lead to other tauopathies with clinical phenotypes different from AD (e.g. frontotemporal dementia). Moreover, it has been shown that formation of NFTs in a transgenic mouse model with P301L Tau mutation was directly triggered after the injection of Aβ fibrils (Götz et al. 2001). Therefore, it has been suggested that NFTs cannot be the initial event in AD course but are likely to occur subsequent to Aβ deposition (Goedert and Jakes 2005; Hutton et al. 1998; Iqbal et al. 2005).

However, it remains unclear, if the amyloid deposition is the crucial event in the development of AD, why it does not correlate with the severity of dementia neither in humans (Price and Morris 1999) nor in transgenic animal models (Moechars et al. 1999; Schmitz et al. 2004). Robust plaque pathology could be observed in otherwise cognitively normal individuals with no signs of dementia (Pimplikar 2009; Snowdon 2003; Villemagne et al. 2008). It has been shown that plaques in human patients are present years before memory deficits appear and that plaque deposition is almost at maximal levels by the time of diagnosis (Jack et al. 2010; Morris and Price 2001; Price et al. 2009). This discovery indicates that further plaque deposition is not connected to the progression of the disease. Moreover, in contrast to human AD patients, in many AD mouse models memory deficits and other pathological alterations occur long before plaques can be detected (Lesné et al. 2008; Walsh and Selkoe 2007).

1.6.3.2 Intracellular amyloid hypothesis

The first report on intracellular Aβ was published in Masters et al. (1985). Later it was shown, that Aβ can be found intracellularly in pyramidal neurons of the hippocampus, entorhinal cortex prior to formation of plaques and NFTs (Fernández-Vizarra et al. 2004; Gouras et al. 2000). Among these discoveries, intraneuronal Aβ has been also found in young patients with Down syndrome without showing amyloid plaques (Gyure et al. 2001; Mori et al. 1992). Further, it has been shown that decreased intracellular Aβ is accompanied by increased plaque pathology and may therefore contribute to the development of extracellular plaques (Gyure et al. 2001; Gouras et al. 2010). The fact that intracellular Aβ aggregation precedes the formation of plaques and NFTs indicates that it could be one of the earliest pathological events in the AD brain (Wirths and Bayer 2012; Wirths et al. 2004).

With the growing evidence that intraneuronal Aβ may be a key contributor in the development of AD, the amyloid cascade hypothesis was modified and ***intracellular Aβ accumulation*** was suggested as the triggering event of neurodegenerative processes in the

brain (Haass and Selkoe 2007; Wirths et al. 2004) (Figure 1.10). The authors suggest that intraneuronal Aβ can originate from intra- and extracellular space. Besides intracellular cleavage of APP, the reuptake of Aβ from the extracellular space may be the second mechanism. In this case, amyloid plaques might serve as a reservoir for neurotoxic Aβ-oligomers, which can affect synaptic structure and plasticity (Wirths et al. 2004).

Several receptors, which may enable the reuptake of Aβ, have been suggested. Among them, there are Apolipoprotein E receptors, nicotinic acetylcholine (nAChR) receptors, integrins and N-methyl-D-aspartate (NMDA) receptors as well as advanced glycation end product receptors.

Accumulation of $A\beta_{42}$ inside neurons has a variety of severe consequences (Mohamed and Posse de Chaves 2011). Intraneuronal Aβ is believed to be responsible for the cell functional failure through mitochondrial arrest (Rhein et al. 2009), following by neuroinflammation and oxidative stress (Mohandas et al. 2009). *In vitro* experiments showed that in neuronal cell culture intracellular Aβ can be localized in endosomes, lysosomes and multivesicular bodies almeida(Almeida et al. 2006; Mohamed and Posse de Chaves 2011; Runz et al. 2002; Takahashi et al. 2004). Accumulation of Aβ in lysosomes causes the loss of lysosomal impermeability and leads to leakage of lysosomal contents such as proteases and cathepsins resulting in apoptosis and necrosis (Ditaranto et al. 2001; Liu et al. 2010; Nixon 2005). It was also shown that intraneuronal oligomeric Aβ species are involved in the disruption of fast axonal transport (Pigino et al. 2009).

The important role of intracellular Aβ in AD was confirmed in a variety of animal models (Bayer and Wirths 2008; Casas et al. 2004; Christensen et al. 2008; Jawhar et al. 2012; Oddo et al. 2003; Shie et al. 2003; Wirths et al. 2001). The toxicity of intracellular Aβ was demonstrated in *in vitro* studies: in cultured rat cortical neurons, apoptosis was triggered by the presence of $A\beta_{1\text{-}42}$ inside of the cells (Kienlen-Campard et al. 2002). The correlation between intraneuronal Aβ and neuron loss reinforced the modified amyloid hypothesis (Casas et al. 2004; Christensen et al. 2008; Jawhar et al. 2012). The relevance of intracellular Aβ has been demonstrated in mouse models of AD that show very little or no extracellular amyloid deposition but behavioral deficits and neuron loss in hippocampus (Bouter et al. 2013a; Bouter et al. 2014; Wittnam et al. 2012). Other mouse models, which develop plaques, showed also strong neuron loss in regions where Aβ accumulates intracellularly (Christensen et al. 2010; Jawhar et al. 2012; Oakley et al. 2006).

Taken together, all studies mentioned above indicate a key role of intracellular Aβ rather than extracellular Aβ depositions in the etiology and pathogenesis of AD.

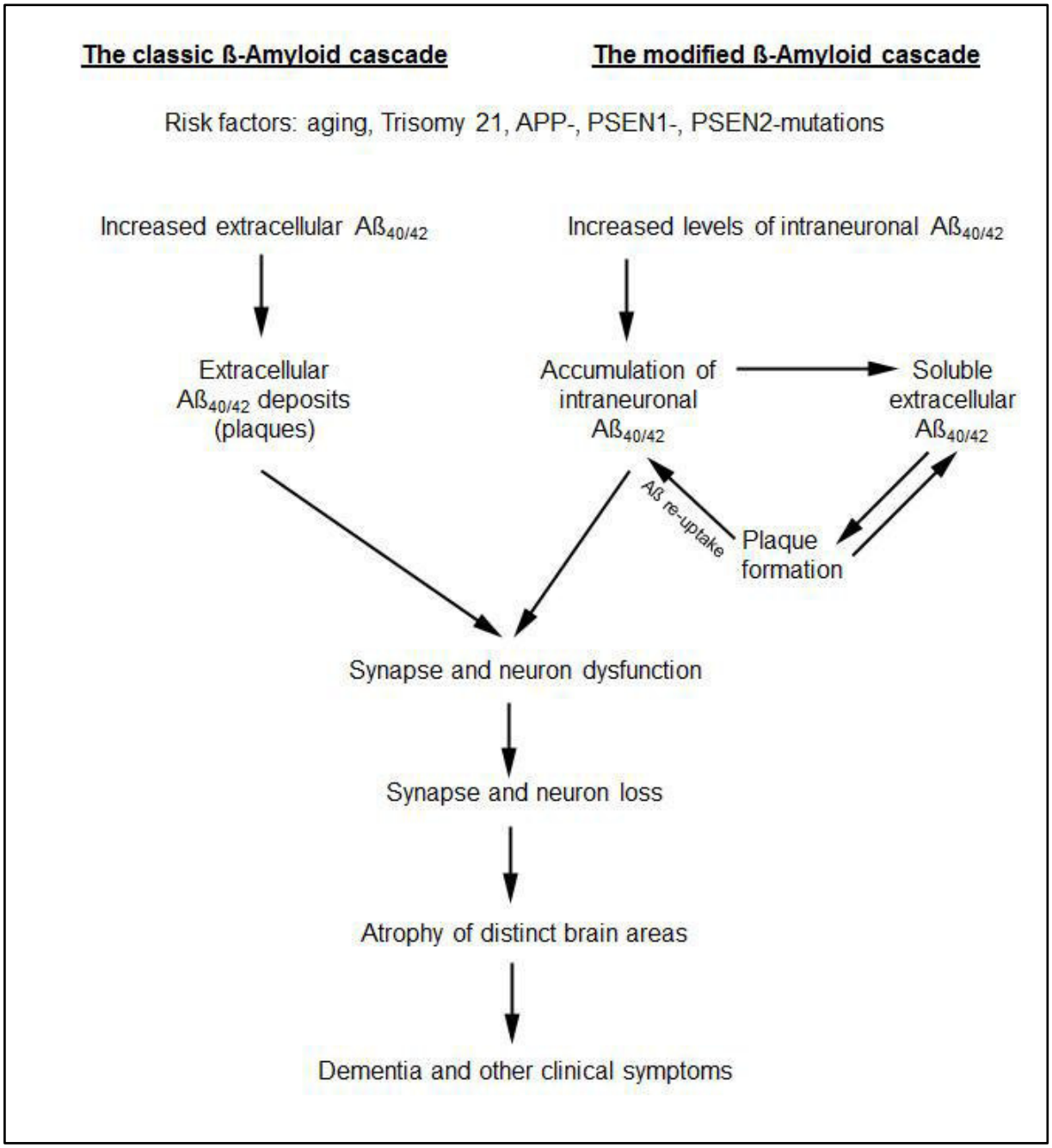

Figure 1. 10: Representation of the classic (on the left side) and the modified (on the right side) amyloid cascade hypothesis. The classic amyloid cascade hypothesis states that extracellular deposition of Aβ is the main event in the pathogenesis of AD. In contrast, according to the modified intracellular amyloid hypothesis, the intraneuronal accumulation of Aβ is the crucial event triggering pathological events in Alzheimer's disease. Figure modified after Wirths et al. 2004, p. 515, p. 517.

1.6.4 Amyloid-beta aggregation

Soluble Aβ monomers produced via amyloidogenic pathway have a high propensity to polymerize and aggregate, forming insoluble Aβ fibrils and later plaques. Monomeric Aβ can form dimers, trimers and higher level homo-oligomers (Benilova et al. 2012). The majority of Aβ appears in forms from dimers to octamers. Soluble oligomers possess a non-fibrillar structure that remains stable in aqueous buffers even after high speed centrifugation (Walsh and Selkoe, 2007). At some point, when a certain threshold is reached, oligomers start to polymerise. They self-associate into protofibrils and later to mature fibrils, which are the major component of plaques. Protofibrils can also dissociate back into lower level species (Roychaudhuri et al. 2009; Walsh and Selkoe 2007) (Figure 1.11). Not all Aβ species show the same biochemical properties and therefore have different tendencies to form fibrils. For instance, $A\beta_{1\text{-}42}$ has a very high propensity to aggregate *in vitro* and *in vivo* (Jarrett et al. 1993). Increased formation and aggregation of neurotoxic Aβ oligomers have been reported to be a critical step in the initiation and development of AD (Haass and Selkoe 2007; Kayed et al. 2003; Li et al. 2009).

The main neurotoxic properties were first attributed to the insoluble deposits (Walsh and Selkoe 2007). However, the toxicity of extracellular insoluble Aβ forms has been questioned since it has been discovered that brains of cognitively and physically intact older people also can display loads of amyloid plaques (Snowdon 1997). It was demonstrated that soluble oligomeric $A\beta_{42}$ correlates better than plaques with the synaptic loss and cognitive decline in AD patients (Bayer and Wirths 2010; McLean et al. 1999). The higher level of Aβ oligomers has been also found to correlate with synaptic dysfunction (Lue et al. 1999; McLean et al. 1999) and with impairment of long-term potentiation (Walsh et al. 2002). Aβ can aggregate and form oligomers *in vitro* due to its amphiphilic structure (Pimplikar 2009). Also it is possible to isolate Aβ oligomers from AD brain extracts and from mice brains of transgenic mouse models (Lesné et al. 2008; Shankar et al. 2008).

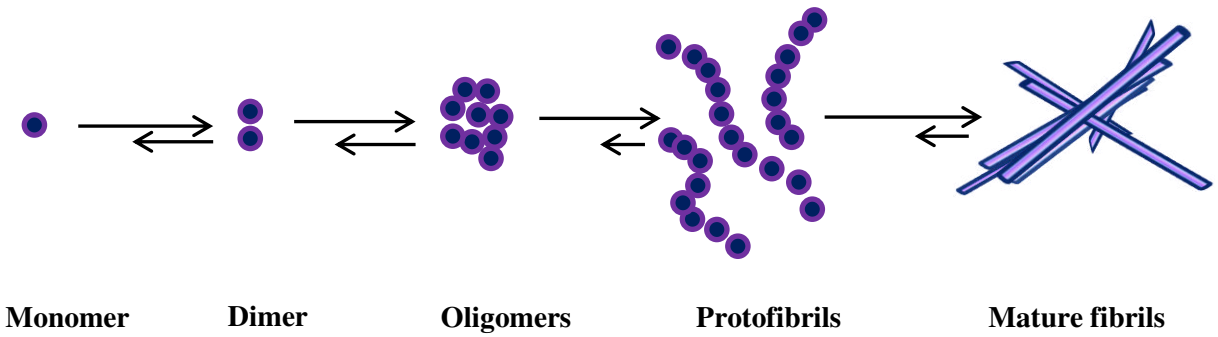

Figure 1. 11: Schematic diagram of Aβ aggregation. Soluble Aβ monomers first form dimers, then oligomers, which subsequently aggregate to protofibrils. Protofibrils assemble to mature amyloid fibrils, which are the main component of plaques. Figure created after Kumar et al. 2011, p. 2263.

1.6.5 Amyloid-beta isoforms

Aβ peptides observed in the human brain are a heterogeneous set of peptides. Aβ isoforms with varying terminal end lengths differ in aggregation propensity, oligomer stability and structure, resistance to proteolytic degradation, and neurotoxicity (Bouter et al. 2013a; Jan et al. 2008; Jarrett et al. 1993; Pike et al. 1995a; Russo et al. 2002; Wirths et al. 2010a).

The two major Aβ forms are $Aβ_{1-40}$ and $Aβ_{1-42}$. It was shown that $Aβ_{1-40}$ is abundant in cerebral vascular amyloid deposits, while $Aβ_{1-42}$ is the major component of neuritic plaques (Iwatsubo et al. 1994; Suzuki et al. 1994). $Aβ_{1-42}$ is more hydrophobic and has a higher propensity to aggregate than $Aβ_{1-40}$. $Aβ_{1-42}$ is considered more neurotoxic and essential for plaque formation (Jarrett et al. 1993; Jan et al. 2008).

Besides $Aβ_{1-40}$ and $Aβ_{1-42}$, there is a variety of C- and N-terminally truncated Aβ forms. These are for instance $Aβ_{1-15/16}$ and $A_{β1-37/38/39}$ (Portelius et al. 2012; Wiltfang et al. 2002) or a range of longer Aβ variants such as $Aβ_{43}$, $Aβ_{45}$, $Aβ_{46}$ and $Aβ_{48}$, that have been identified in cell lines and have also been found in AD mouse models (Esh et al. 2005; Van Vickle et al. 2008; Welander et al. 2009). $Aβ_{43}$ was detected in plaques of human AD patients in at least the same high amount as $Aβ_{40}$ (Welander et al. 2009).

Furthermore, there are various post-translational modifications of Aβ (Kummer and Heneka 2014): pyroglutamylation, phosphorylation and oxidation, as well as nitration, racemization, isomerization and glycosylation. The most important modification of Aβ is probably the formation of pyroglutamate (pGlu)-modified Aβ species. pGlu modifications of Aβ lead to its increased aggregation tendency, disturbed proteolytic degradation and exerts high neurotoxicity (Kummer and Heneka 2014; Kuo et al. 1997; Pike et al. 1995a; Russo et al. 2002; Wirths et al. 2010c). Mori et al. (1992) reported that approximately 15-20 % of all Aβ peptides are N-terminally pyroglutamate-modified. Oxidation and nitration of Aβ happen as a response to inflammation in AD (Heneka et al. 2010) and is supposed to prevent the formation of protofibrils (Hou et al. 2002).

1.6.5.1 N-terminally truncated Aβ

A variety of N-terminally truncated Aβ isoforms has been described (Bayer and Wirths 2014; Masters et al. 1985; Mori et al. 1992; Selkoe et al. 1986; Sergeant et al. 2003). Their ragged N-termini result from different cleavage. It has been shown in *in vitro* experiments that shortening of the N-terminus increases Aβ aggregation (Pike et al. 1995a) which probably promotes plaque formation *in vivo* (Soto et al. 1995). Usually, Aβ is cleaved by BASE1 resulting in a peptide with the aspartate at the first position (Asp-1). BASE1 is also able to cleave Aβ between Tyr-10 and Glu-11 leading to production of $Aβ_{11–x}$ species (Vassar et al. 1999). Several proteases have been suggested to be involved in the production of N-truncated Aβ isoforms (Figure 1.12). However, the exact enzymatic activities mostly remain not well understood (Bayer and Wirths 2014).

It was demonstrated that the metalloprotease Meprin-β is involved in the Aβ cleavage, generating Aβ starting at residue 2 (Bien et al. 2012). A zinc-metalloprotease Neprilysin (neutral endopeptidase or NEP) cleaves Aβ between Arg-2 and Glu-3, Glu-3 and Phe-4, Arg-5 and His-6 (Howell et al. 1995; Iwata et al. 2001; Bayer and Wirths 2014). In addition, it has been reported that myelin basic protein is also able to cleave Aβ between Phe-4 and Arg-5 at the N-terminus of the Aβ-sequence (Liao et al. 2009) as well as plasmin, which produces Aβ species starting at His-6 (Van Nostrand and Porter 1999; Tucker et al. 2000).

Aβ isoforms with ragged N-termini are highly abundant in the human AD brain (Kawarabayashi et al. 2001; Portelius et al. 2010; Saido et al. 1995). For instance, $Aβ_{2-x}$ variants are elevated in brains and reduced in the cerebral spinal fluid of AD patients. It is likely that $Aβ_{2-x}$ are a result of cleavage first by BASE1 and then by aminopeptidase A (Bibl et al. 2012; Wiltfang et al. 2001).

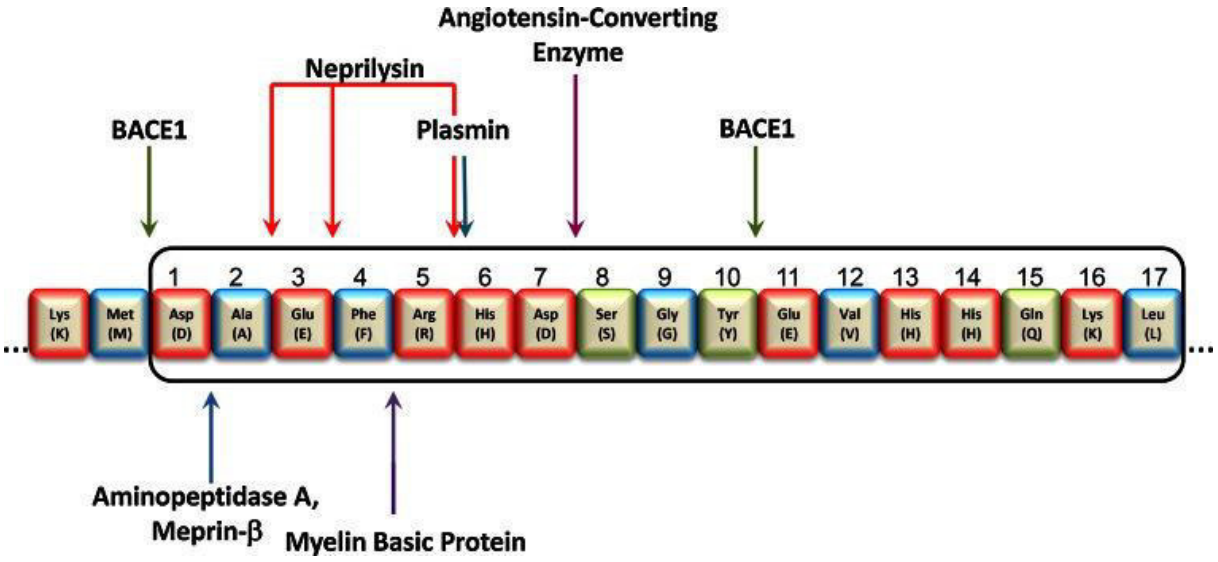

Figure 1. 12: The cleavage sites of enzymes involved in the degradation of full-length Aβ and potential generation of N-truncated Aβ isoforms. Sequence of the first 17 amino acids of the N-terminus of human Aβ is displayed in three-letter and one-letter-code. Colour code for amino acids: *red* indicates amino acids with charged polar side-chains; *green* - with uncharged polar side-chains; blue – hydrophobic non-polar amino acids. Figure from Bayer and Wirths 2014, p.789.

$Aβ_{3-x}$ has been observed in plaques in late-onset AD patients and AD mouse models (Casas et al. 2004; Güntert et al. 2006). It has been shown *in vitro* that Aβ starting with a pyroglutamate-modified residue three (Glu-3), $Aβ_{pE3-x}$, oligomerizes more quickly and forms fibrillar, β-sheet structures more rapidly than $Aβ_{1-x}$ (Harigaya et al. 2000; He and Barrow 1999; Schlenzig et al. 2009). $Aβ_{pE3}$ was found in amyloid plaques in the same amounts as $Aβ_{1-x}$ or even greater. It has been suggested that $Aβ_{pE3}$ accumulation precedes that of other Aβ species (Saido et al. 1995).

$Aβ_{4-x}$ species are one of the first Aβ discovered. They are abundant in AD and vascular dementia (Masters et al. 1985; Lewis et al. 2006) (see 1.6.5.2).

Comparing to $A\beta_{4\text{-}x}$ variants, the knowledge about $A\beta_{5\text{-}x}$ is very poor. It was detected both in AD patients and non-demented controls (Murayama et al. 2007; Sergeant et al. 2003) The role of $A\beta_{5\text{-}X}$ remains still unclear (Bayer and Wirths, 2014).

Apart from $A\beta_{11\text{-}x}$, the largest known amino-truncations are cleavages at Asp-7, Ser-8 and Gly-9. Angiotensin-converting enzyme has been suggested as an enzyme leading to the generation of $A\beta_{8\text{-}x}$ (Kalback et al. 2002; Kummer and Heneka 2014; Sergeant et al. 2003).

N-truncated Aβ species are the main components of amyloid plaques in AD and can also be found intracellularly (Bayer and Wirths 2014; Gouras et al. 2000).

1.6.5.2 Amyloid beta 4-42

Aβ starting at residue four (Phe-4) is abundant in AD. It was discovered in 1985 as a component of brain amyloid deposits (Masters et al. 1985). However, it is still a not well investigated Aβ isoform. There is not enough knowledge about the contribution of $A\beta_{4\text{-}42}$ to the development and progression of AD. After investigation of amyloid depositions in two sporadic AD cases, Masters et al. (1985) reported that 64 % of Aβ peptides from amyloid plaques started with a Phe-4, while, according to (Miller et al. 1993) and (Näslund et al. 1994), $A\beta_{4\text{-}42}$ is less abundant in AD brains. Another finding supports the results from Masters et al. (1985) showing that $A\beta_{4\text{-}42}$ is one of the major fractions in hippocampus and cortex of human AD patients (Portelius et al. 2010). Aβ4-42 was found in cotton wool plaques in FAD patients with a V261I PSEN1 mutation (Miravalle et al. 2005) as well as in the amyloid deposits in patients with familial Danish dementia and vascular dementia (Tomidokoro et al. 2005; Lewis et al. 2006).

In a recent study from our group, it was shown that $A\beta_{4\text{-}42}$ is as toxic as $A\beta_{pE3\text{-}42}$ and $A\beta_{1\text{-}42}$. Moreover, treatment of wild-type mice with intraventricular $A\beta_{4\text{-}42}$ injection caused significant working memory deficits. These deficits were similar to the memory impairments induced through $A\beta_{pE3\text{-}42}$ and $A\beta_{1\text{-}42}$ species (Bouter et al. 2013a). It was also demonstrated that $A\beta_{4\text{-}42}$ aggregates to oligomers more rapidly than longer N-truncated Aβ variants (Pike et al. 1995a) and shows a strong fibrillization propensity (Haupt et al. 2012).

All these studies indicate that $A\beta_{4\text{-}42}$ plays an important role in the development and progression of AD.

1.7 Mouse models of Alzheimer's disease

1.7.1 Mouse models and their relevance in AD research

After the discovery of mutations that lead to familial Alzheimer's disease, a variety of transgenic mouse models has been generated. The majority of these models are based on the overexpression of human APP and/or PSEN1/2 with at least one familial AD mutation.

The neuropathological alterations, cognitive and motor impairments in these models resemble main characteristics of human AD to a greater or lesser extent. Transgenic animal models differ from each other in their phenotype. That can be attributed to the genetic background, different mutations, different promotors and the transgene doses (Elder et al. 2010). The first successful AD transgenic mouse model based on mutant APP was the PDAPP model. It was reported by Games et al. (1995) and contained human APP with the Indiana mutation (V717F) under the control of the platelet derived growth factor promotor. PDAPP mice exhibit age-dependant extracellular plaque pathology, dystrophic neurites, gliosis, synapse loss and age-related learning impairment without neuron loss (Chen et al. 2000; Dodart et al. 2000; Games et al. 1995; Irizarry et al. 1997; Reilly et al. 2003)

Another murine model based on the APP mutation is the Tg2576 model. These transgenic mice overexpress human APP with the Swedish mutation (K670N/M671L) under the control of a hamster prion protein promotor (Hsiao et al. 1996). It is one of the most widely studied mouse models of AD, which displays age-dependant plaque pathology together with axonal impairments and gliosis similar to PDAPP mice. Significant plaque pathology in Tg2576 mice can be observed at the age between 11 and 13 months. Behavioural impairments can be observed prior to the massive plaque depositions (Elder et al. 2010; Holcomb et al. 1998; Hsiao et al. 1996).

Several other APP-based models, which contain one or more FAD mutations have been developed and characterized. In summary, they show elevated Aβ levels, extracellular plaque pathology, gliosis, dystrophic neurites and signs of inflammation. Furthermore, behavioural and cognitive impairment have been also frequently observed (Chishti et al. 2001; Elder et al. 2010; Games et al. 2006; Sturchler-Pierrat et al. 1997). Recently, a human APP knockin mouse model has been described. It develops amyloid pathology and memory deficits expressing the mutant APP at endogenous levels (Nilsson et al. 2014).

The mutations in presenilins 1 and 2 also have been taken to develop transgenic mouse models. Mouse models harbouring these transgenes exhibit elevated levels of $A\beta_{X\text{-}42}$ but do not develop plaque pathology (Cavanaugh et al. 2014). The absence of plaques can be explained through the lower levels of $A\beta_{X\text{-}42}$ in the PSEN-mutated lines comparing to APP models, or through the different solubility of human and murine Aβ (Jankowsky et al. 2007).

In order to intensify the pathological phenotype, mouse models with a combination of multiple APP mutations or APP and PSEN mutations were created. The APP/PS1ΔE9 model (Borchelt et al. 1997), the APP/PS1KI model (Casas et al. 2004) and the 5XFAD model (see 1.7.1) are well-characterized. They display an earlier onset of pathology and more abundant plaque deposition (Elder et al. 2010). Research on these mouse models significantly contributed to the better understanding of neuron loss, behavioural phenotype and the role of intracellular Aβ (Casas et al. 2004; Christensen et al. 2010; Jawhar et al. 2012; Oakley et al. 2006; Oddo et al. 2003; Wirths and Bayer 2012; Wirths et al. 2009). Intraneuronal Aβ has been reported in a variety of mouse models including Tg2576, 3xTg, APP/PS1K1 and 5XFAD (Billings et al. 2005; Casas et al. 2004; Oakley et al. 2006; Oddo et al. 2003; Takahashi et al. 2013). Several mouse models also showed high correlation between intraneuronal Aβ accumulation and neuron loss (Wirths and Bayer, 2012).

The relevance of mutant APP/PS transgenic models is sometimes called in question, because the mutations are clearly associated only with FAD, which makes up barely 1 % of entire AD cases. Regarding this fact, it is reasonable to create a model for sporadic AD. However, until now there has been nearly no success in these endeavours. Unfortunately, rodents do not develop AD pathology spontaneously during the aging process (Sarasa and Pesini 2009). Nevertheless, one non-transgenic mouse model with some important pathological characteristics of sporadic AD including memory impairment has been described. The mice were subjected to intracerebroventricular (icv) injection of streptozotocin (STZ) in order to induce insulin resistant state in the brain. Diabetes mellitus type II as a probable risk factor for AD could be observed in this icv-STZ mouse model. Indeed, the icv-STZ mice show cognitive deficits, decreased energy metabolism, altered synaptic proteins, increased hyperphosphorylated Tau and signs of neuroinflammation in the brain (Chen et al. 2012; Chen et al. 2013; de la Monte and Wands 2008; Salkovic-Petrisic et al. 2006).

Besides the icv-STZ, a novel model of sporadic AD, Tg4-42, was recently generated in our group (Bouter et al. 2013a) (see 1.7.2). This model supports the hypothesis that intracellular soluble aggregates of Aβ play a crucial role in AD.

Numerous species such as rats, rabbits, dogs and primates have been proposed for research purposes in the field of AD, but mice still remain the most commonly used vertebrates in this sphere. Mice have many advantages such as a relatively short reproductive cycle, large litters and easy handling (Sarasa and Pesini, 2009). Mouse models of AD are very important for the research and contribute a lot to the understanding of etiology and pathology of Alzheimer's disease. Unfortunately, the entire clinical and neuropathological pictures of familial or sporadic AD in humans cannot be fully reconstructed in any of the available models. Anyway, showing pathological features of AD such as amyloid deposition, neuron loss, aggregation of phosphorylated Tau and behavioural and/or memory deficits, mice models reflect various aspects of the disease and therefore help us to get more insights in its pathogenesis and enable making next steps towards developing a therapy against it.

1.7.2 The 5XFAD mouse model

The 5XFAD mouse model was first described by Oakley et al. (2006). This model is a double transgenic APP/PS1 mouse model expressing in total five familial AD mutations (5XFAD): human APP (isoform APP_{695}) carrying the Florida (I716V), London (V717I) and Swedish (K670N/M671L) mutations, and mutant PSEN1 (M146L; L286V), all under the control of a murine Thy-1 promoter (Vidal et al. 1990; Moechars et al. 1996; Oakley et al. 2006) (Figure 1.13). These mice display stable germline transmission and coinheritance of the APP and PSEN-1 transgenes over multiple generations, so these mice can be bred as if they are a single transgenic mouse model (Eimer and Vassar 2013; Oakley et al. 2006).

In the 5XFAD mouse model, the Florida, London and the two PSEN mutations lead to increased production of $A\beta_{x\text{-}42}$ species, whereas the Swedish mutation leads to elevated levels of total Aβ.

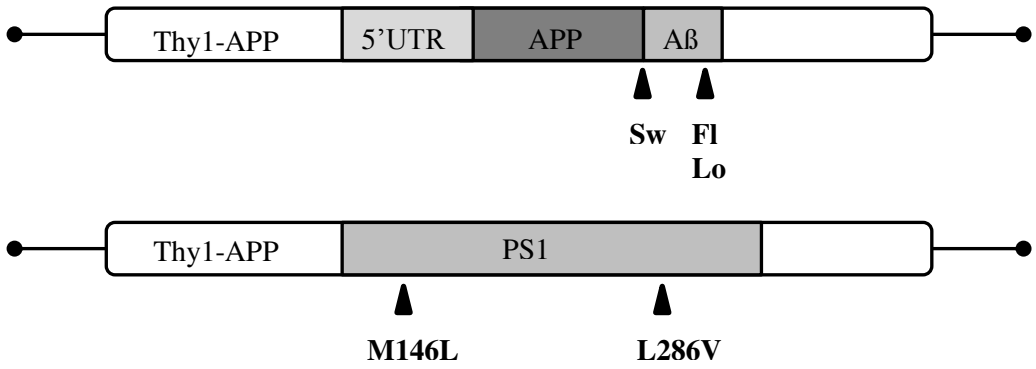

Figure 1. 13: 5XFAD transgenes. Schematic diagram of 5XFAD APP and PS1 transgenes. FAD mutations are indicated by arrowheads. Abbreviations: Sw, Swedish mutation; Lon, London mutation; Fl, FL mutation; 5'UTR, 5' untranslated region. Small grey rectangles represent Thy1 exons. Coding regions of APP and PS1 are displayed by larger rectangles inserted into Thy1 exon 2 of the mouse Thy1 transgene expression cassette. Figure created after Oakley et al. 2006, p. 10131.

5XFAD mice develop their pathological phenotype rapidly and display key features of AD. Plaque pathology starts at the age between two and three months and is accompanied by gliosis. These characteristics massively increase with age (Oakley et al. 2006). 5XFAD mice also display extracellular amyloid depositions in spinal cords at three months of age (Jawhar et al. 2012). Besides extracellular plaques, intracellular Aβ can be observed in the cortical Layer 5 and the subiculum starting at the age of six weeks (Eimer and Vassar 2013) (Oakley et al. 2006). Intraneuronal Aβ appears prior to plaque formation and correlates well with neuron loss in these brain regions (Oakley et al. 2006; Jawhar et al. 2012). Furthermore, the 5XFAD mouse shows significant neuron loss. Design based stereology revealed a significant neuron loss in the pyramidal neurons of the Layer 5 cortex and the subiculum starting at the age of 9 months (Eimer and Vassar 2013). Interestingly, neither intraneuronal Aβ nor neuron loss could be found in the CA1 region of the hippocampus, despite presence of amyloid plaques in this region (Jawhar et al. 2012). The 5XFAD model also develops age-dependant synaptic degeneration and dystrophic neurites (Oakley et al. 2006). Dystrophic changes in these structures are thought to be associated with the deposition of extracellular Aβ plaques (Zhang et al. 2009b).

Moreover, 5XFAD mice develop age-dependant progressive behaviour deficits. At the age of roughly 6 months, 5XFAD mice show behavioural impairment and working memory deficits. Reference memory impairments are found the age of 12 month (Oakley et al. 2006; Jawhar et al. 2012). The mice also demonstrate decreased anxiety. Impairments in the Y-maze, Morris water maze, the conditioned taste aversion task and contextual fear conditioning were observed in the 5XFAD model (Devi and Ohno 2010; Devi and Ohno 2013; Jawhar et al. 2012; Ohno et al. 2006). Besides cognitive impairments, 5XFAD mice also exhibit motor deficits, which can be linked to extensive spinal cord pathology, including dystrophic neurites and intracellular Aβ accumulation (Jawhar et al. 2012).

5XFAD is a widely used model in Alzheimer's research. Several groups used this model in preclinical studies investigating possible treatments and their effects on the progression of cognitive impairment (Aytan et al. 2013; Bhattacharya et al. 2014; Cho et al. 2014; Fiol-deRoque et al. 2013; Hillmann et al. 2012; Wirths et al. 2010a).

1.7.3 The Tg4-42 mouse model

The Tg4-42 model is a transgenic mouse model for sporadic Alzheimer's disease, which was generated in our group (Bouter et al. 2013a; Bouter et al. 2013b). This model is the first model, which expresses N-truncated human $A\beta_{4\text{-}42}$ without any other mutation and still develops age-dependant neuronal loss and behavioural deficits (Bouter et al. 2013a). $A\beta_{4\text{-}42}$, as mentioned in 1.6.5, is one of a variety of N-terminally truncated Aβ species identified in the AD brain and may be a particularly abundant Aβ isoform in the human brain (Masters et al. 1985; Lewis et al. 2006; Portelius et al. 2010).

The genetic construct of Tg4-42 contains the $A\beta_{4\text{-}42}$ sequence fused to the murine thyrotropin-releasing hormone (TRH) signal peptide under the control of the neuronal Thy-1 promoter (Figure 1.14). This allows the Aβ to be secreted into the extracellular space in a neuron-specific manner (Bouter 2014).

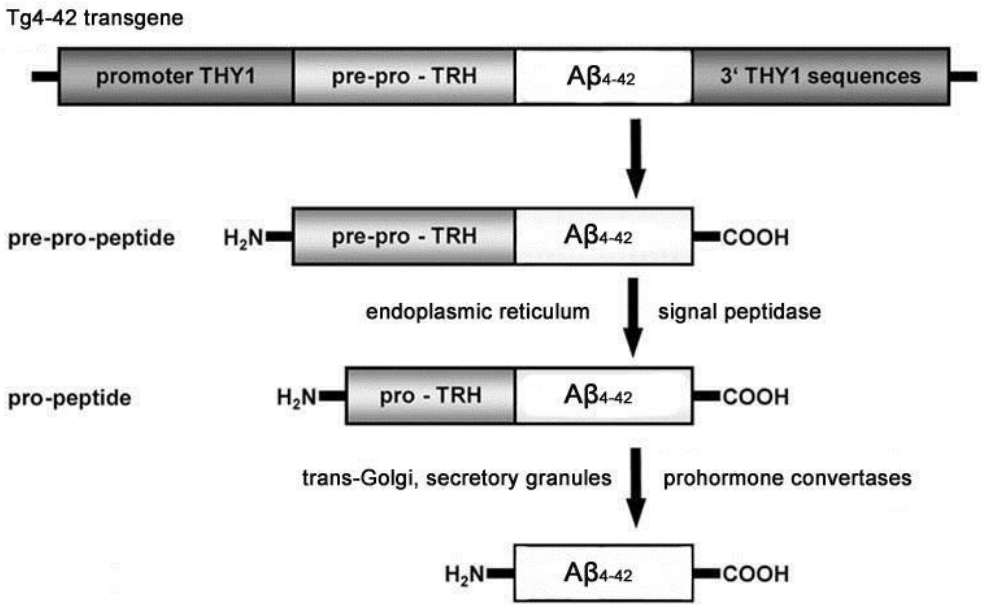

Figure 1. 14: Genetic construct of the Tg4-42 transgene mouse. The murine Thy1 promoter drives the neuronal expression of the pre-pro-TRH-$A\beta_{4\text{-}42}$ fusion peptide. In the construct of Tg4-42, Aβ4-42 is fused to pre-pro-TRH for product liberation within the secretory pathway. An N-terminal signal sequence guides the pre-pro-TRH-$A\beta_{4\text{-}42}$ fusion peptide into the endoplasmic reticulum, where signal peptidases cleave it and release the pro-TRH-Aβ4-42 peptide. Prohormone convertases in the trans-Golgi and secretory vesicles cleave the rest of the TRH signal peptide to release $A\beta_{4\text{-}42}$. Figure modified from Alexandru et al. 2011, p. 12792 and Bouter 2014, p. 31.

The transgene expression is highest in the hippocampus, mainly in the CA1 pyramidal cell layer, and is detectable starting at the age of two months. $A\beta_{4\text{-}42}$ could be also found in occipital cortex, piriform cortex, striatum, and superior colliculus. The Tg4-42 model does not express human APP, so the effects of the $A\beta_{4\text{-}42}$ peptide are not disturbed by the influence of APP or other cleavage products.

The secreted $A\beta_{4\text{-}42}$ is a highly toxic peptide. It has strong propensity to forms soluble, neurotoxic aggregates triggering neuron death in primary cortical neurons. The Tg4–42

mice do not develop any extracellular amyloid plaques; NTFs are also absent. However, the Tg4–42 mice develop age- and dose-dependent neuronal loss in the hippocampus CA1 region of hemizygous and homozygous mice, as determined by unbiased stereology. In addition, reactive microglia and astrocytes can be found in the hippocampus starting at the age of two months. Behaviourally, the Tg4-42 mice show strong spatial reference memory deficits as observed in the Morris water maze test. Memory deficits are age-dependent and are not detectable at the age of three months. The impaired spatial reference memory in Tg4-42 is compatible with AD-typical age-dependent cognitive decline (Bouter et al. 2013a; Bouter et al. 2013b; Bouter et al. 2014).

Taken together, the Tg4-42 model is a unique mouse model for sporadic AD. All other transgenic models that develop all pathologies mentioned above have at least one mutation. The Tg4-42 model has no mutations and represents the first mouse model expressing exclusively N-truncated $A\beta_{4\text{-}42}$. Therefore, the effects of this peptide can be investigated without interference from other human APP products. Another advantage of this model is the absence of amyloid plaques, which do not correlate well with cognitive decline in AD patients.

1.8 Therapeutic approaches

1.8.1 Pharmacological interventions

At the present time, there is no cure for Alzheimer's disease. All current medications aim at mitigating the symptoms. Available drugs such as memantine and different acetylcholinesterase inhibitors do not target the underlying cause, they only bring some relief.

The first class of drugs used in the treatment of AD became available in the late 90s. It was ***acetylcholinesterase inhibitors***, which aim at the effects of degeneration of cholinergic neurons and subsequent exhaustion of the neurotransmitter acethylcholine. It is known that the cholinergic system is involved in memory and behavior processes (Bartus et al. 1982). Thus, it is held that impairments in cholinergic neurons in AD lead to memory and cognitive deficits (Terry and Buccafusco 2003).

There are different drugs that inhibit the catalytic function of acetylcholinesterase enzyme that degrades acetylcholine in the synaptic cleft, but the opinions are controversial about their efficacy (Pepeu and Giovannini 2009). Among them, donepezil (Rogers and Friedhoff 1996), rivastigmine (Polinsky 1998), and galantamine (Raskind et al. 2000) are routinely prescribed. There is some evidence that the acetylcholinesterase inhibitors can temporarily delay the progression of the disease or moderate the symptoms. The result of the treatment depends on how early the treatment is started and on the responsiveness of the patients. However, none of these three drugs can cure or prevent the progression of the clinical symptoms (Bullock et al. 2005; Bullock and Dengiz 2005; Rogers et al. 1998; Wallin et al. 2011).

Another type of drugs used in AD treatment is memantine. Memantine is a ***non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist*** that is prescribed to patients with moderate to severe AD (Tariot et al. 2004). It protects the nerve cells against glutamate, which is released in excess by AD-damaged brain cells (Parsons et al. 2007). Memantine has some beneficial effects in patients with AD and is used in combination with cholinesterase inhibitors (McShane et al. 2006; Orgogozo et al. 2002; Parsons et al. 2013; Peskind et al. 2006). All pharmacological interventions mentioned above showed similar limited effects in humans and mice (Yuede et al. 2007), supporting the validity of animal models for AD research. Furthermore, a variety of clinical trials focused on the reduction of neuroinflammation were performed (Meinert et al. 2009), as well as immunization against toxic Aβ species (Wisniewski 2009).

The intracellular amyloid cascade hypothesis suggests that modulating the production or increasing the clearance of Aβ could open new possibilities in treatment of AD. It was tried to inhibit or modulate the enzymes that are involved in the amyloidogenic pathway. However, up to date substrates specific but non-toxic γ-secretase inhibitors have not been developed. Inhibitors of β-secretase are also at a very early developmental stage.

Therefore, the research in the field of active and passive immunization gains a higher importance (Lannfelt et al. 2014).

Most of the immunization approaches against Aβ have substantial disadvantages such as negative side effects or a lack of efficacy in humans. The remarkable effects of ***active immunization*** against Aβ in the PDAPP mouse model led to the initiation of clinical trials of active immunization (Dodart et al. 2002; Janus et al. 2000; Kotilinek et al. 2002; Morgan et al. 2000). Except some patients in the first clinical phase of study failed to develop detectable antibody titers, no adverse effects were observed (Schenk 2002). Unfortunately in the clinical phase 2a, severe side effects of active immunization such as CNS inflammation and pulmonary embolism (Ferrer et al. 2004; Nicoll et al. 2003) occurred and the study was halted. The side effects have been attributed to an autoimmune response (Orgogozo et al. 2003).

Many researchers see the future in ***passive immunization*** strategies (Hock et al. 2002). Several antibodies have been proposed for passive immunization. For clinical trials, they have been

humanized to avoid immune-responses in human patients. Passive immunization against Aβ has been successfully tested in murine models during the past years. It was shown that peripherally administered antibodies are able to clear Aβ from murine brain (Bard et al. 2003; DeMattos et al. 2001; Frost et al. 2012; Wilcock et al. 2003; Wilcock et al. 2004a; Wilcock et al. 2004b; Wilcock et al. 2006).

However, passive immunization also caused side effects such as microhemorhages (DeMattos et al. 2012; Lannfelt et al. 2014; Pfeifer 2002; Racke et al. 2005; Schroeter et al. 2008; Wilcock et al. 2004b; Wilcock et al. 2006). Some antibodies showed an effect in the reduction of the cognitive decline but without statistical significance. However, none of these antibodies have been proven to substantially ameliorate the deficits in AD or influent progression of symptoms (Lannfelt et al. 2014).

In the recent years, the role of soluble toxic Aβ aggregations and some of its N-truncated isoforms in the pathogenesis of AD have attracted a lot of attention. Now it is

widely accepted that $A\beta_{pE3\text{-}X}$ may play an important role in AD. Another abundant N-truncated species in human AD brain is $A\beta_{4\text{-}X}$ (Bouter et al. 2013a; Bouter et al. 2014). The first studies of anti-$A\beta_{pE3\text{-}X}$ immunotherapy in mice had promising outcomes (DeMattos et al. 2012; Frost et al. 2012; Wirths et al. 2010a). Therefore, N-truncated Aβ isoforms became of a great interest as possible therapeutic targets. Passive immunotherapy against Aβ species is an important topic of ongoing research and remains a promising option to develop an efficient treatment of AD.

However, considering the fact that there are still no drugs that can cure AD, the importance of ***preventing approaches*** grows immensely. There are a variety of studies confirming that a healthy and physically active lifestyle together with cognitive stimulating activities and a Mediterranean diet reduce risk for Alzheimer's disease.

1.8.2 Environmental interventions

A variety of epidemiological studies underline the beneficial effects of different environmental factors, such as physical activity (Hillman et al. 2008; Lautenschlager et al. 2008), participation in leisure activities (Crowe et al. 2003; Akbaraly et al. 2009), intellectual and cultural activities (Cummings et al. 1998) and a healthy balanced diet (Feart et al. 2015) (Willett 2006) show indisputable benefits in older adults at risk for AD (Knopman 2009).

Feart et al. (2015) reviewed recent epidemiolocal studies concerning the Mediterranean diet. The Mediterranean diet consists of vegetables, fruits, low fat meats such as fish and poultry, polyunsaturated fats such as olive oil, and unrefined cereals. The Mediterranean diet has protective effect on cognitive health, including a reduced risk of Alzheimer's disease and cognitive impairment. Overall, it was suggested that the Mediterranean diet might exert a long-term beneficial effect on brain functioning (Willett 2006).

One of the widely accepted explanations of beneficial effects of environmental interventions is the ***cognitive reserve*** (CR) hypothesis. According to Steffener and Stern (2012), cognitive reserve is an ability to make flexible and efficient use of available brain reserve when performing tasks. CR has been most often estimated using IQ and education. Other variables have also been used (e.g. literacy, occupational complexity, participation in leisure activities, cohesion of social networks, personality variables, socioeconomic status, and aerobic fitness). Individuals with higher cognitive reserve tend to have better clinical outcomes for any level of pathology and brain reserve[3] (Steffener and Stern 2012; Stern 2002; Stern 2006; Stern 2009; Hillman et al. 2008). It was suggested that environmental interventions promote a better CR that helps coping with pathological changes in brain during the Alzheimer's disease (Akbaraly et al. 2009; Albert et al. 1995).

[3] Steffener and Stern (2012) have suggested that two types of reserve contribute to maintaining functioning in the presence of brain changes or insult: brain reserve and cognitive reserve. Brain reserve is the reserve, which derives from brain size or neuronal cell number (e.g. larger brains can sustain more insult before clinical deficit emerges, because sufficient neural substrate remains to support normal function).

A growing body of evidence suggests that also lifelong bilingualism may delay the onset of AD by several years (Gold 2015). It was shown in epidemiological studies that individuals who speak two languages on a regular basis since childhood tend to develop clinical AD symptoms at an older age than monolinguals. Thus, bilingualism is very interesting as a potential form of CR, primary because it appears to be a primarily environmental factor for which neither special education nor intelligence is needed (Bialystok et al. 2007; Alladi et al. 2013; Gold 2015; Lawton et al. 2015).

Physical exercises promote reconfiguration of brain networks, which is essential in learning, memory, and executive functions (Foster 2015). In wild-type rodents, it was shown that physical activity increases cell proliferation and adult hippocampal neurogenesis (Blackmore et al. 2009; Couillard-Despres et al. 2005; van Praag et al. 1999), and improves learning (Stranahan and Mattson 2008). In spite of evidence for the importance of physical activity, 74% of adults in the United States have sedentary lifestyle and do not meet the recommended guideline of at least 30 minutes of moderate-intensity physical daily activity. Recent epidemiological studies indicate that children are also growing increasingly unfit, and that these lifestyle factors are related to an earlier onset of a variety of chronic diseases. Numerous studies also showed that participation in physical activity has been associated also with the decrease of a number of physical (e.g. cardiovascular disease, cancer diseases, obesity) and mental (e.g. dementia, depression and anxiety) disorders during the life. (Hillman et al. 2008).

The ***environmental enrichment*** (EE) is an experimental setting in which the animals are housed in cognitively and physically stimulating conditions. It is used in mouse models to simulate stimulating human living conditions (e.g. social interactions, sensory and motor stimulation).

The pathological phenotype of several transgenic mouse models of AD exposed to environmental enrichment was significantly improved. Decreased Aβ levels and inflammation markers were found in the environmentally enriched Tg2576 mice (Nichol et al. 2007, S. 2; Nichol et al. 2008; Stranahan et al. 2012). Spatial memory was improved and Alzheimer's disease-like pathology was alleviated in senescence-accelerated prone-8 (SAMP8) mice (Dong et al. 2012). Enriched environment also ameliorated performance in cognitive tasks in the PS1/PDAPP (Costa et al. 2007). In the TgCRND8 mice, a decrease of amyloid deposition has been observed after EE (Adlard et al. 2005). It was also shown in TgCRND8 mice that cognitive and physical stimulation increases neurogenesis in hippocampus to wild type levels. Furthermore, EE prevents astrogliosis in hippocampus of adult PDAPP-J20 and 3xTg transgenic mice (Beauquis et al. 2013; Rodríguez et al. 2013). EE induced also beneficial effects on working memory in 3xTg mice (Blázquez et al. 2014). In addition, EE rescues adult neurogenesis in the APP23 mouse model of AD (Mirochnic et al. 2009). However, EE failed to ameliorate SGZ neurogenesis in APP/PS1K1 as well as neuropathological alterations and memory deficits (Cotel et al. 2012). Positive effects due to EE have also been reported in sporadic models of Alzheimer such as APOE ε4 mice (Nichol et al. 2009). It was suggested that EE improves diminished cellular plasticity in AD brain via enhancing the brain capacity to better compensate for neurodegeneration (Herring et al. 2009). Due to its neurogenic effect, EE was considered to be a potential therapeutic approach in AD (Schaeffer et al. 2009; Um et al. 2008).

1.9 Aims of the study

The experimental work presented in the following pages aimed to further investigate two mouse models of Alzheimer's disease: Tg4-42 and 5XFAD. Another goal of my doctoral study was to determine the effects of environmental intervention on histopathological features of the Tg4-42 mice.

The present study consists of two projects. The aim of ***the first project*** was to further characterize the new model for sporadic AD, ***Tg4-42*** expressing $A\beta_{4-42}$, which was recently developed in our group. Because of the variety of typical for AD symptoms displayed by this unique model, it is of great interest to evaluate the effects of physical, social and sensorial stimulation.

Therefore, groups of Tg4-42 transgenic females were exposed to EE at an early age and in a continuous manner. They were subsequently tested for hippocampus-dependent memory, motor abilities, anxiety levels (projects of my colleagues), and their cerebral tissue was thoroughly analysed using unbiased stereology for neurogenesis and neuronal loss in the dentate gyrus (present study). Groups of Tg4-42 mice housed under enriched environmental conditions were compared to the age-matched Tg4-42 and wild-type mice from the standard housing. The dentate gyrus region was chosen, because it is located in the centre of the hippocampal formation, which is severely affected in AD. The hippocampus is thought to be involved in memory and learning processes. In addition, the dentate gyrus is a unique region, in which adult neurogenesis takes place. Adult hippocampal neurogenesis is a process involving the continuous generation of newborn neurons in in the subgranular zone of the dentate gyrus. Mounting evidence has suggested that hippocampal neurogenesis contributes to some forms of hippocampus-dependent learning and memory and therefore may be a potential therapeutic target in AD treatment.

The second project aimed at investigation the gene dosage dependence of the neuropathological features in the widely used mouse model for familial Alzheimer's disease ***5XFAD***.

Therefore a homozygous 5XFAD strain was created to elevate transgene expression and Aβ production. In order to evaluate the gene dosage-dependent effect on the extent of amyloidosis and axonal impairments, hemizygous and homozygous mice were compared. Sagittal brain sections of hemizygous and homozygous 5XFAD mice at 2, 5 and 9 months of age were stained with an Aβ[N] antibody, which detects an N-terminal Aβ epitope, and the amyloid burden was quantified in the cortex, hippocampus (hippocampal region (CA and DG), retrohippocampal region (subiculum)) and thalamus. In addition, axonal swellings located in the pons and spinal cord were counted in order to evaluate the extent of axonal pathology.

2. MATERIALS AND METHODS

2.1 Animals

2.1.1 General considerations

All animals used in this study were of the species Mus musculus. Wild-type mice were of the inbred strain C57Bl/6J (Jackson Laboratories, Bar Harbor, ME, USA). The mice were housed under specific-pathogen-free conditions at the central animal facility of the Universitätsmedizin Göttingen (the Göttingen University Medical Centre) in standardized laboratory cages (described in 2.1.4). Food pellets and fresh tap water have been provided ad libitum. All animals were handled according to the guidelines of the "Society for Laboratory Animals Science" (GV-SOLAS) and the guidelines of the "Federation of European Laboratory Animal Science Association" (FELASA). The experiments were approved by the "Landesamt für Verbraucherschutz und Lebensmittelsicherheit" (LAVES) Niedersachsen (Lower Saxony State Office for Consumer Protection and Food Safety). All efforts were made to reduce the number of animals used for this study and to minimize their suffering. Individuals that suffered from massive loss of weight (>20 %) or other severe conditions such as blind eyes were sacrificed immediately and excluded from analyses.

Unless otherwise specified, males and females were used for analyses. The mouse models used in this study are listed in table 2.1.

Table 2. 1: Mouse models used in the current study

Mouse model	*Gene*	*Protein*	*Mutations*	*Promotor*
Tg 4-42	APP	N-truncated Aβ 4-42	absent	murine Thy-1
5xFAD	APP	APP695	Swedish (K670N, M671L), Florida (I716V), London (V717I)	murine Thy-1
	PSEN1	PS1	M146L, L286V	murine Thy-1

2.1.2 Tg4-42 transgenic mice

The Tg4-42 model is a transgenic mouse model, which reflects the sporadic form of Alzheimer's disease. This model was previously generated in our lab. Tg4-42 mice do not have any mutations but show neurological deficits due to transgenic overexpression of human N-truncated-A$\beta_{4\text{-}42}$ fused to the murine TRH signal peptide under the control of the neuronal Thy-1 promoter (Bouter et al. 2014; Wittnam et al. 2012) (see 1.7.2). The homozygous Tg4-42 mouse line (Tg4-42$_{hom}$) was also generated in our lab and has been used in the current study (see table 2.2). In order to produce potential homozygous mice, hemizygous Tg4-42 mice were crossed. In order to validate homozygosity, quantitative Real-Time PCR was performed on DNA extracted from mouse tails followed by backcrossing of presumptive homozygous mice with wild-type mice to verify the true homozygosity. After homozygosity of the males and females was confirmed, they were re-mated to generate Tg4-42$_{hom}$ mice.

Table 2. 2: Tg4-42 and wild-type mice used in the current study

Genotype	*Living conditions*	*Age (months)*	*Sex*	*Number of animals used*	*Left hemisphere*
Tg4-42$_{hom}$	EE	6	female	11	+
Tg4-42$_{hom}$	SH	6	female	4	+
Tg4-42$_{hom}$	SH	6	male	4	+
Wild-type (C57Bl/6J)	SH	6	female	8	+

2.1.3 5XFAD transgenic mice

The 5XFAD model was initially generated and described by (Oakley et al. 2006) (see 1.7.1). 5XFAD mice used in this study were maintained on a C57Bl6/J genetic background (Jackson Laboratories, Bar-Harbor, ME, USA) (Jawhar et al. 2012). 5XFAD transgenic mice express five familial AD mutations (5XFAD): human amyloid precursor protein (APP695) carrying the Swedish (K670N, M671L), London (V717I) and Florida (I716V) mutations and human presenilin-1 (PSEN1) carrying the M146L/L286V mutations, all under the control of the murine Thy1-promoter (Moechars et al. 1996; Oakley et al. 2006; Vidal et al. 1990)**.** These five mutations are known to cause familial AD in humans.

To generate a homozygous 5XFAD line, heterozygous 5XFAD mice were crossed. The offspring was analysed using quantitative Real-Time PCR of genomic DNA obtained from the mouse tails for human APP with murine APP as a calibrator. 5XFAD mice, which showed an approximately doubled human APP gene dosage compared to 5XFAD$_{hem}$ mice, were identified as potential 5XFAD$_{hom}$ mice. Homozygosity of potential 5XFAD$_{hom}$ mice was confirmed by backcrossing with wild-type mice before they were used for further breeding (Richard et al. 2015).

Only female hemizygous and homozygous 5XFAD mice were used for the neuropathological analyses at 2 and 5 months of age. For the analysis at 9 months of age, only male mice were used (table 2.3).

Table 2. 3: 5XFAD mice used in the current study

Genotype	*Age (months)*	*Sex*	*Number of animals used*	*Right hemisphere*	*Spinal cord*
$5XFAD_{hom}$	2	female	4	+	-
	5	female	7	+	-
	9	male	4	+	+
$5XFAD_{hem}$	2	female	6	+	-
	5	female	8	+	-
	9	male	3	+	+
	9	male	3	+	-

2.1.4 Housing conditions

2.1.4.1 Standard housing

From weaning until the age of 8 weeks, all mice were housed in standardized laboratory cages (individually ventilated cages (IVC), 32 x 16 x 14 cm) in groups of up to five individuals. The mice assigned to the standard housing (SH) living condition were kept in these cages in groups of four to five females till the date of sacrificing. Males were kept alone in separate cages in order to avoid hierarchy-related conflicts and possibly resulting injuries. This way of keeping promotes social interactions without hierarchy-related aggressive behaviour (Fitchett et al. 2005). Sawdust bedding and some nesting material were provided.

2.1.4.2 Environmental enrichment

The 8 week-old Tg4-42 mice that were assigned to the environmental enrichment (EE) living condition were transferred to rat cages (55 cm x 34 cm x 20 cm) in groups of four to five females. In contrast to SH-cages, in the larger EE-cages mice had access to a variety of objects such as running wheels, plastic toys, tunnels and igloos, which were changed and spatially rearranged every week in order to stimulate spatial cognition (O'Keefe and Nadel 1978). Running wheels were accessible all the time to promote physical activity (van Praag et al. 1999).

2.2 Isolation of genomic DNA and genotyping of animals

2.2.1 DNA isolation from mouse tails

All mice used for the neuropathological analyses in this study were genotyped. For genotyping, genomic DNA was isolated from mouse tail biopsies. Per sample, 500 µl of lysis buffer [100 mM Tris/ HCl (pH 8.5), 5 mM EDTA, 0.2% (w/v), sodium dodecyl sulfate (SDS), and 200 mM NaCl] and 5 µl Proteinase K (20 mg/ml stock) were added. Samples were incubated for 20 hours at 55°C in a Thermomixer Compact under gentle agitation (350-450 rpm). Afterwards, samples were centrifuged for 20 minutes at 4°C and 17,000 rpm. The supernatant was transferred into a new 1.5 ml Eppendorf tube containing 500 µl cold isopropanol and the pellet was discarded. Samples were vortexed then until the DNA precipitate was visible. This was followed by another 10 minutes of centrifugation at 13,000 rpm at room temperature (RT). After centrifugation, the supernatant was discarded and the DNA containing pellet was washed in 500 µl 70% (v/v) ice-cold ethanol. The solutions were centrifuged one more time at 13,000 rpm for 10 minutes at RT. After the supernatant was discarded, the pellet was left to dry at 37°C in a Thermomixer Compact for 45 minutes. The pellet was then dissolved in 30 µl molecular grade water and left overnight at 56°C in a Thermomixer Compact before being stored at 4°C.

2.2.2 Determination of DNA concentration and purity

The DNA concentration of each sample was determined via photometry. As a blank for the photometry settings, 80 µl of molecular grade water was used. An amount of 2 µl of each sample was diluted with 78 µl molecular grade water in a Uvette® 220-1600 nm. The A260/A230 absorbance ratio and the A260/A280 absorbance ratio were measured in the photometer for each DNA sample. DNA samples with A260/A230 and A260/A280 > 1.8 were considered sufficiently pure (Barbas et al. 2007). Afterwards, samples were diluted to a concentration of 20 ng/µl in molecular grade water.

2.2.3 Genotyping of animals using polymerase chain reaction (PCR)

The extracted and diluted genomic DNA from mouse tails, was used for genotyping. 5XFAD and Tg4-42 mice carrying the transgene were identified using conventional polymerase-chain-reaction (PCR).

PCR reactions were performed in a LabCycler using 0.2 ml PCR tubes. The DNA (2 µl) was given into a PCR reaction tube and the prepared master mix (18 µl) was added. A master mix consisted of 10x reaction buffer, 25 mM magnesium chloride, Taq polymerase (5 U/µl), dNTPs, molecular grade water and the respective primers. All primers were used at a concentration of 10 pmol/µl (1:10 dilution of the 100 pmol/µl primer stock prepared in

ddH_2O). All primers were purchased from Eurofins (Ebersberg, Germany) as intron-spanning validated primer pairs. The primers used in this study are given in the table 2.4.

Reaction mixes for the PCR and cycling conditions are listed in the table 2.5, table 2.6 and table 2.7.

Table 2. 4: List of primers used for mouse genotyping.

Primer	***Sequence (5′ → 3′)***
Aβ3-42 for	GTGACTCCTGACCTTCCAG
Aβ3-42 rev	GTTACGCTATGACAACACC
hAPP for	GTAGCAGAGGAAGAAGTG
hAPP rev	CATGACCTGGGACATTCTC

Table 2. 5: Reaction mix for Tg4-42 PCR.

Reagent	***Volume [µl]***
DNA (20 ng/µl)	2.0
Aβ3-42 for2 primer	1.0
Aβ3-42 rev2 primer	1.0
dNTPs (2 mM)	2
MgCl2 (25 mM)	1.6
10x reaction buffer	2
Molecular grade water	10.2
Taq polymerase (5 U/µl)	0.2
Total volume per sample	20

Table 2. 6: Reaction mix for 5XFAD PCR.

Reagent	***Volume [µl]***
DNA (20 ng/µl)	2.0
hAPP for primer	0.5
hAPP rev primer	0.5
dNTPs (2 mM)	2
MgCl2 (25 mM)	3.2
10x reaction buffer	2
Molecular grade water	9.6
Taq polymerase (5 U/µl)	0.2
Total volume per sample	20

Table 2. 7: PCR cycling program for genotyping Tg4-42 and 5XFAD mice.

Step	*Temperature [°C]*	*Duration [s]*
1	94	180
2	94	45
3	58	60
4	72	60
5	Repetition of steps 2-4 (35 times)	
6	72	300
7	4	∝

2.2.4 DNA agarose gel electrophoresis

The PCR products were loaded on agarose gels to identify transgene animals using gel electrophoresis.

1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid) was necessary for the gel electrophoresis. At first, 10x TBE buffer was made as follows: 108 g Tris and 55 g boric acid were dissolved in 900 ml ddH_2O. Then 40 ml of 0.5 M ethylenediaminetetraacetic acid (pH 8.0) was added to the solution. The volume was adjusted to 1 l with ddH_2O. The solution was diluted 1:10 in ddH_2O to obtain 1x TBE buffer.

To prepare the gel, 100 ml of 1x TBE buffer was added to 2 g agarose and boiled in a microwave at 560 W until the agarose was completely dissolved.

Afterwards, 3 µl ethidium bromide (10 mg/ml) was added to the liquid gel and the gel was casted in a casting tray with a comb to form wells. Any air bubbles were removed. After the gel was cooled down, the comb was carefully removed. The gel was then positioned in an electrophoresis chamber filled with 1x TBE buffer. The samples were treated as follows: 10 µl of the PCR product was mixed with 1 µl 10x agarose gel sample buffer and loaded into a well. The gel was run in a horizontal electrophoresis chamber connected to a power. For size indication, one well was filled with 5 µl of the 100 bp DNA ladder and run in TBE buffer for approximately 45 minutes at 120 V constant current. The DNA in the gel was visualized under a UV light (366 nm) using the Gel Doc 2000 (Biorad, Hercules, CA, USA) and analysed with the software program Quantity One (Version 4.30; Biorad).

2.2.5 Quantitative Real-Time PCR Genotyping of 5XFAD mice

The homozygous 5XFAD mice were identified through quantitative Real-Time PCR (qRT-PCR) using a MX3000P Real-Time Cycler with 10 ng of genomic DNA per reaction. For the quantification of the PCR product, the SYBR-green based DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) containing ROX as an internal reference dye was used. The DNA dilutions were mixed with the qPCR reaction

mix in 200 µl PCR tubes and briefly centrifuged. The reaction mix and cycling protocol are given in tables 2.8 and 2.9. The primers used for qRT-PCR are given in the table 2.10.

Table 2. 8: List of primers (Eurofins, Ebersberg, Germany) used for qRT-PCR

Primer	***Sequence (5′ → 3′)***
hAPP for	GTAGCAGAGGAAGAAGTG
hAPP rev	CATGACCTGGGACATTCTC
mAPP-for	TCTTGTCTTTCTCGCCACTGGC
mAPP-rev	GCAGTCAGAAGTTCCTAGG

Table 2. 9: Reaction mix for 5XFAD qRT-PCR

Reagent	***Volume [µl]***
DNA (20 ng/µl)	2.0
hAPP for or mAPP-for primer	0.5
hAPP rev or mAPP-rev primer	0.5
Master Mix	10
ROX	0.2
Molecular grade water	6.3

Table 2. 10: qRT-PCR cycling program for detecting 5XFAD$_{hom}$ mice

Step	***Temperature [°C]***	***Duration [s]***
1	95	600
2	95	15
3	64	20
4	72	30
5	Repetition of steps 2-4 (40 times)	
6	95	60
7	55	30
	95	30

The reaction was performed in duplicates in separate tubes for each pair of primers. All data were collected using the MxPro Mx3000P software (Stratagene, Santa Clara, CA, USA).

Average C_T values were determined from the duplicates. C_T value (threshold cycle value) is the cycle value at which a statistically significant increase in fluorescence is first detected. Relative quantification was performed using murine APP as a reference gene for normalization. The transgene levels of human APP (hAPP) were normalized to those of murine APP (mAPP) and calibrated to a selected 5XFAD animal with confirmed genotype using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001):

$$(1) \qquad Relative\ Amount_{Gene} = 2^{-\Delta\Delta C_T}$$

For an animal (q), the level of hAPP gene expression was normalized to the expression of mAPP as a reference gene and calibrated to an animal with confirmed genotype (cb). - $\Delta\Delta C_T$ is calculated as follows (2) and (3):

$$\Delta C_T = C_{T,hAPP} - C_{T,mAPP} \quad (2)$$

$$-\Delta\Delta C_T = -\left(\Delta C_{T,q} - \Delta C_{T,cb}\right) \quad (3)$$

Figure 2.1 gives an example, how animals have being identified as potentially homozygous or heterozygous 5XFAD animals.

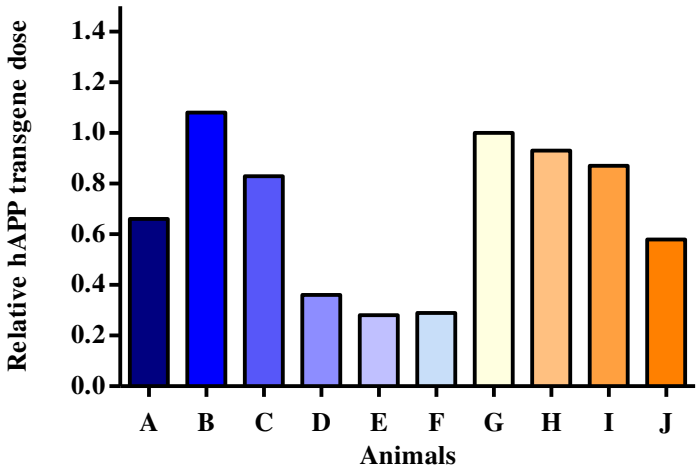

Figure 2. 1: An example for Real-Time-PCR genotyping of 5XFAD mice. Human APP gene doses in the animals A, B, C, G, H, I and J were at least twice as high in comparison to the animals D, E and F. Mice G, H, I and J were previously confirmed to be homozygous. Mice A, B, C with unknown genotype were identified as homozygous and mice D, E and F were considered hemizygous. All mice were calibrated here to one random chosen homozygous mouse (here mouse G). Source: own research.

2.3 Tissue collection and preservation

Tissue was collected and preserved in different ways depending on the following analysis.

For neuronal stereology (cresyl violet staining) and immunostaining on frozen free-floating sections the mice were deeply anesthetized by a mixture of ketamine and xylazine diluted in molecular-grade water. The mice were anesthetized by an intraperitoneal injection at a dosage of 100 mg/kg ketamine and 10 mg/kg xylazine body weight.

Thereafter a fixation was done by transcardial perfusion through the left ventricle with 30 ml ice-cold 0.01 M Dulbecco's phosphate buffered salt solution (PBS) in order to clear the body from blood. Afterwards perfusion was continued by 30 ml of 4% paraformalde-

hyde (PFA) dissolved in 0.01 M PBS. Discolouring of the liver indicated a successful perfusion.

The brains and spinal cords were carefully removed and brains were divided at the midline. The left hemispheres were post-fixed for at least 2 hours in 4% phosphate-buffered formalin at 4°C and cryoprotected overnight in a 30% sucrose solution. When the brain was sunken to the bottom it was quickly frozen directly on a block of dry ice and stored deep-frozen at -80°C. The right hemispheres were embedded in paraffin as described below.

In order to collect sections for neuronal stereology and for the quantification of doublecortin-positive neurons in free-floating frozen sections, the fixed frozen brains were taken out of the 80°C-freezer and left at -20°C for at least 1 hour followed by embedding in a soluble glycol and resin compound. Immediately after this each frozen hemisphere was cut in 10 series of 30 µm-thick coronal sections on a cryostat. Every tenth section throughout the brain was collected in a small glass container. The glass containers with the sections were stored frozen at -80°C until further processing.

For the immunohistochemical staining of the 5XFAD mice, they were deeply anesthetized as described above, then also transcardially perfused with 30 ml ice-cold 0.01 M PBS in order to remove the blood cells and hereby decrease background staining in immunohistochemistry. Some animals were sacrificed without perfusion via CO_2 anesthetization followed by cervical dislocation. After the mouse cadavers were decapitated, brains and spinal cords were carefully dissected on ice and brains were divided at the midline. The tissue (right hemispheres and spinal cords) was placed into embedding cassettes and preserved by fixation in 4% paraformaldehyde in 0.01 M PBS at 4°C for at least 3 days, then dehydrated and immersed in paraffin using a TP1020 Automatic Tissue Processor (Leica). Then the tissues were embedded in paraffin blocks using an EG1140 H Embedding Station (Leica). For immunohistochemistry, 4 µm sagittal sections of the right hemispheres and transversal sections of spinal cords were produced by cutting paraffin blocks with a microtome and transferring tissue sections onto glass slides in a 50-54°C hot water bath. The slides were dried at 37°C for at least 10 hours before they were used for immunohistochemistry.

2.4 Histological Stainings

2.4.1 Cresyl violet staining

The frozen section series comprising every tenth section throughout the brain (one section series from each animal) were rehydrated in cold 0.01 M PBS solution in order to remove organic glue. Then, they were carefully mounted on glass slides. When dried, the sections were stained with a cresyl violet acetate solution (Powers and Clark 1955).

At first the sections were washed twice for 10 minutes in a 0.04 M natriumacetate trihydrate/0,1% acetic acid solution (1B). Thereafter the sections were delipidated for 20

minutes in a 0,025% Triton X-100/75% ethanol solution (3B), washed two times for 10 minutes in 1B and stained two times for 8 minutes in 0.01% cresyl violet in 1B solution. Then, the sections were washed three times for 1 minute in 1B solution, followed by 3 minutes in 100% ethanol, 10 minutes in isopropanol, and twice for 5 minutes in xylene before being embedded in a Roti®Histokit mounting medium. Then a cover slip was added to each slide. The solutions required for cresyl violet staining and the staining protocol are listed in the tables 2.11 and 2.12.

Table 2. 11: Solutions required for cresyl violet staining

Solution	***Description***
Buffer stock solution (1A)	13.61 g natriumacetate trihydrate in 100 ml ddH_2O
Buffer work solution (1B)	40 ml buffer stock solution (1A) + 9.6 ml 100 % acetic acid + 950.4 ml ddH_2O
Cresyl violet staining solution (2)	0.1 g cresyl violet in 1 l buffer working solution (1B). Stir for 30 min. Let rest overnight. Filter right before use.
Delipidation stock solution (3A)	2 ml Triton X-100 + 98 ml ddH_2O. Stir for 1 h.
Dilipidation work solution (3B)	2,5 ml delipidation stock solution (3A) + 50 ml ddH_2O + 150 ml 100 % ethanol

Table 2. 12: Cresyl violet staining protocol

Step	***Description***
1. Delipidation	2 x 10 min in buffer work solution (1B) 20 min in delipidation work solution (3B) 2 x 10 min in buffer work solution (1B)
2. Staining	2 x 8 min in cresyl violet solution (2)
3. Dehydration	3 x 1 min in buffer working solution (1B) 3 min in 100 % ethanol 10 min in isopropanol
4. Embedding	2 x 5 min in xylene 2 drops of xylene-based mounting medium Roti®Histokit. A cover glass should be firmly pressed on top of glass slide, carefully pushing away the air bubbles when necessary.

2.4.2 Immunostainings

2.4.2.1 DAB immunohistochemistry of paraffin embedded section

For visualising the Aβ depositions and axonal spheroids, diaminobenzidin (DAB) immunohistochemistry was used.

The glass slides with the dried, paraffin embedded sections were deparaffinized twice for 5 minutes in xylene and rehydrated in baths of decreasing ethanol concentrations (10

minutes in 100% ethanol, 5 minutes in 95% ethanol, 1 minute in 70% ethanol, 1 minute in ddH_2O). Endogenous peroxidases were blocked for 30 minutes in 0,3% (v/v) hydrogen peroxide in 0.01 M PBS and antigen retrieval was carried out by boiling the sections for 10 min in 0.01 M citrate buffer (pH 6.0) in a microwave (to expose the epitopes) after which the sections were left for 15 minutes at RT to cool down. Membrane permeabilization was achieved by washing three times for 5 minutes in 0.01 M PBS containing 0.1% (v/v) Triton X-100, followed by antigen retrieval in 88% formic acid for 3 minutes (only for Aβ staining, to improve the visualization of the intracellular species of Aβ). Then the sections were circled with a lipid pen. Unspecific binding sites were blocked by one hour treatment with 4% (w/v) low-fat dried milk powder and 10% (v/v) fetal calf serum (FCS) in 0.01 M PBS. Afterwards, the blocking solution was removed (without extra washing) and the sections were incubated overnight in a humid chamber at room temperature with primary antibodies of desired concentration diluted in 0.01 M PBS containing 10% (v/v) FCS. The next day the sections were washed three times for 5 minutes in 0.01 M PBS containing 0.1% (v/v) Triton X-100 and were incubated with secondary antibodies at 37°C for 1 hour. After washing, the sections were incubated for 1 hour in the respective biotinylated secondary antibody. The secondary antibody was diluted in 0.01 M PBS containing 10% FCS to the desired concentration (see table 2.16). Then the sections were washed three times for 5 minutes in 0.01 M PBS and incubated 90 minutes at 37°C in Avidin-biotin complex (ABC) solution containing 0.01 M PBS, 10% (v/v) FCS and solutions A (1:100) and B (1:100) from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) (further "Vectastain Kit") using the ABC method. This solution was prepared according to the instructions of the manufacturer at least 30 minutes before use and stored at 4°C. After incubation in the ABC-solution the slides were washed three times for 5 minutes with 0.01 M PBS in order to remove the ABC solution.

The staining was finally visualized by 3,3'-Diaminobenzidine (DAB) as chromogen providing reddish-brown colour. The DAB stock solution consisted of 25 mg/ml DAB in 50 mM Tris/HCl (pH 7.5). The sections were incubated for up to 3 minutes in the DAB staining solution, which consisted of 100 µl DAB stock solution, 5 ml 50 mM Tris/HCl, and 2.5 µl 30% H_2O_2 added immediately before use. Afterwards, the sections were washed three times for 5 minutes in 0.01 M PBS. The DAB staining was counterstained with hematoxylin for 40 seconds and put for 5 minutes under running tap water. Thereafter, the sections were dehydrated in baths of increasing ethanol concentrations (1 minute in 70% ethanol, 5 minutes in 95% ethanol, and 10 minutes in 100% ethanol) and xylene (two times for 5 minutes). This was followed by embedding in Roti®Histokit mounting medium before a cover glass was firmly pressed on each slide.

For the Aβ plaque load quantification (see 2.5), the sections were not counterstained in order to avoid prominent background.

Solutions required for DAB immunohistochemistry and the DAB-Immunostaining protocol for paraffin-embedded sections are listed in the tables 2.13 and 2.14.

The antibodies used for this immunostaining are listed in 2.4.2.3.

Table 2. 13: Solutions required for DAB immunohistochemistry

Solution	*Description*
0,01 M PBS solution	Concentrate (PBS 10x Dulbecco's) diluted in ddH_2O
0,01 M citrate buffer (pH 6.0)	2.1 g citric acid monohydrate per liter of ddH_2O. The pH was adjusted by NaOH.

Table 2. 14: DAB-Immunostaining protocol for paraffin-embedded sections on glass

Step	*Description*
1. Deparaffinization	2 x 5 min in xylene
2. Hydration	Baths of decreasing concentrations of ethanol: 10 min 100% ethanol 5 min 95% ethanol 1 min 70% ethanol 1 min ddH_2O
3. Blocking the endogenous peroxidase activity	30 minutes-incubation in 0,3% (v/v)H_2O_2 in 0.01 M PBS (200 ml 0.01 M PBS + 2 ml 30% (v/v)H_2O_2 1 min ddH_2O
4. Antigen retrieval	To expose the epitopes: Treating sections in the microwave at 800 W in 0.01 M citrate buffer (pH 6.0) for 2-3 min until boiling, then heating at 80 W for 8 min. 15 min cool down at room temperature
5. Washing and cell membranes permeabilisation	1 min ddH_2O 15 min 0.01 M PBS + 0.1% (v/v) Triton X-100
6. Unmasking intracellular Aβ epitopes	3 min incubation in 88% formic acid (to improve the visualization of the intracellular species of Aβ) 1 min 0.01 M PBS
7. Washing	5 min 0.01 M PBS
8. Blocking unspecific binding	1 hour incubation at RT in solution made of 0.01 M PBS, 10% (v/v) FCS, and 4% (w/v) low-fat dried milk powder. Incubation by circling sections on glass with lipid pen.
9. Primary antibody incubation	After unspecific blocking without washing incubation overnight at room temperature with the antibody in a 0.01 M PBS/10% (v/v) FCS solution.
10. Washing	The day after, 3 x 5 min washing in 0.01 M PBS + 0.1% (v/v) Triton X-100
11. Secondary antibody incubation	1 hour incubation in the corresponding biotinylated secondary antibody diluted in 0.01 M PBS/10 % (v/v) FCS, at 37°C.
12. Washing	3 x 5 min in 0.01 M PBS
13. ABC incubation	90 min incubation at 37°C in 0.01 M PBS + 10% (v/v) FCS + Solution A (1:100) + Solution B (1:100) from the Vectastain Kit. Solution was prepared at least 30 min before use and stored at 4°C.
14. Washing	3 x 5 min in 0.01 M PBS
15. DAB-Staining	Incubation in 0.5 mg/ml DAB in 50mM Tris/HCl (pH 7.5) for a few minutes: 5 ml 50 mM Tris/HCl (pH 7.5) + 100 µl DAB stock solution + 2.5 µl 30% H_2O_2 (added just before use). The evolution of

Step	*Description*
	the staining intensity was checked under a microscope and the reaction was stopped by putting the slides in a bath of 0.01 M PBS.
16. Washing	3 x 5 min in 0.01 M PBS
17. Counterstaining	40 s incubation in hematoxylin 1 min in in ddH_2O 5 min under running tap water 1 min ddH_2O
18. Dehydration	Baths of increasing ethanol concentrations. 1 min 70% ethanol 5 min 95% ethanol 10 min 100% ethanol
19. Embedding	2 x 5 min xylene Embedding in a xylene-based mounting medium Roti®Histokit. A cover glass should be firmly pressed on top of glass slide, carefully pushing away the air bubbles when necessary.

2.4.2.2 Free-floating immunohistochemistry

The embedding procedure and the harsh treatments (for example high temperatures) adversely affect some epitopes. That is why immunostainings in paraffin sections are inapplicable for detection of antigens with sensitive epitopes. Nevertheless, in most cases those antigens can be visualized by immunostaining in free-floating frozen sections. That was the case for the neurogenesis marker doublecortin (DCX) for which a specific protocol has been applied. The following DAB-Immunostaining protocol for free-floating frozen sections was taken up from (Cotel 2009) and modified.

In this study, DCX was used to visualize new-born neurons and reflect neurogenesis in Tg4-42$_{hom}$ mice (Figure 2.2). DCX is a microtubule-associated protein that is expressed in all migrating neuronal precursors of the developing central nervous system (Couillard-Despres et al. 2005). Initially, doublecortin positive neurones were found in the subventricular zone where new neurons start migration to the olfactory bulbs, and in the subgranular zone of the dentate gyrus (Lledo et al. 2006) (see 1.5.5).

The brain treatment was done as described above in 2.3. The frozen section series comprising every tenth section throughout the brain (one section series from each animal) were rehydrated for 10 minutes in 4°C 0.01 M PBS solution in order to remove organic glue. The cold 0.01 M PBS was used to avoid breaks in the integrity of the slices due to heat-shock. Then the sections were transferred into Netwells™ using cold 0.01 M PBS as transfer liquid (all sections from each glass container were put in separate Netwells™). All of the following incubation and washing steps were carried out in Netwells™ (Figure 2.3) on a rotating plate.

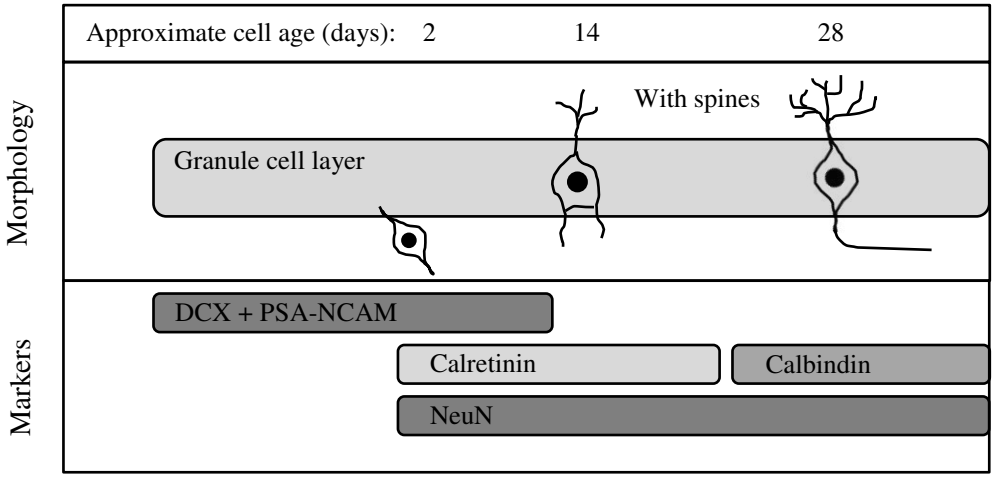

Figure 2. 2: Hippocampal granule cells expressing different markers. Abbreviations: DCX, doublecortin; GAD, glutamate decarboxylase; NeuN, neuronal nuclear antigen; PSA-NCAM, polysialic acid–neuronal cell adhesion molecule. Figure created after Lledo et al. 2006, p. 181.

At first, the sections were quenched for 30 minutes in a solution of 0.01 M PBS with 0,3% (v/v) H_2O_2 for the purpose of blocking endogenous peroxidase activity. The sections were subsequently washed three times for 10 minutes in 0.01 M PBS/0.1% (v/v) Triton X-100 followed by incubation for one hour at room temperature in a solution of 4% (w/v) low-fat dried milk powder and 10% (v/v) FCS in 0.01 M PBS in order to block unspecific binding sites. Then the blocking solution was removed and the primary antibody (DCX) diluted (1:500) in 0.01 M PBS containing 10% (v/v) FCS was added into Netwells®. The sections were incubated overnight at room temperature. The next day the sections were washed three times for 10 minutes in 0.01 M PBS containing 0.1% (v/v) Triton X-100 and incubated with a secondary antibody diluted as above at room temperature for two hours. Then the sections were washed three times for 10 minutes in 0.01 M PBS and incubated for 90 minutes at RT in a Avidin-biotin complex (ABC) solution from the Vectastain Kit using the ABC method (see 2.4.2.1).

After washing the staining was visualized by 3,3'-Diaminobenzidine. The DAB staining solution was made as described in 2.4.2.1. The sections were incubated for 5 minutes in the DAB staining solution. Afterwards, the sections were washed three times for 10 minutes in 0.01 M PBS.

After washing, stained sections were mounted onto glass slides, then carefully unfolded with a thin brush and left dry overnight at RT.

The next day the sections were counterstained with hematoxylin. This was done as follows. At first, the glass slides were put for 10 minutes in 0.01 M PBS for hydration. Then the slides were counterstained with hematoxylin for 40 seconds, dipped shortly in ddH_2O and put for 5 minutes under running tap water. Thereafter, the sections were dehydrated in baths of increasing ethanol concentrations as described in 2.4.2.1 and subsequently embedded in Roti®Histokit mounting medium before a cover slip was added to each slide.

The DAB-Immunostaining protocol for free-floating frozen sections is recapitulated in table 2.15.

The antibodies used for this immunostaining are listed in 2.4.2.3.

Table 2. 15: DAB-Immunostaining protocol for free-floating frozen sections

Step	***Description***
1. Organic glue removal and rehydration	10 min in 0.01 M cold 0.01 M PBS in which the sections are moved into Netwells™. 10 min 0.01 M PBS for hydration.
2. Blocking the endogenous peroxidase activity	30 minutes-incubation in 0,3% (v/v)H_2O_2 in 0.01 M PBS (200 ml 0.01 M PBS + 2 ml 30% (v/v)H_2O_2
3. Washing	3 x 10 min in 0.01 M PBS + 0.1% (v/v) Triton X-100 1 min in 0.01 M PBS
4. Blocking unspecific binding	1 hour incubation at room temperature in solution made of 0.01 M PBS, 10% (v/v) FCS, and 4% (w/v) low-fat dried milk powder.
5. Primary antibody incubation	After unspecific blocking, without washing, incubation overnight at room temperature with the antibody in a 0.01 M PBS/10% (v/v) FCS solution.
6. Washing	3 x 10 min in 0.01 M PBS
7. ABC incubation	90 min incubation at 37°C in 0.01 M PBS + 10% (v/v) FCS + Solution A (1:200) + Solution B (1:200) from the Vectastain Kit. Solution was prepared at least 30 min before use and stored at 4°C.
8. Washing	3 x 10 min in 0.01 M PBS
9. DAB-Staining	Incubation in 0.5 mg/ml DAB in 50mM Tris/HCl (pH 7.5) for 5 min: 5 ml 50mM Tris/HCl (pH 7.5) + 100 µl DAB stock solution + 2.5 µl 30% H_2O_2 (added just before use). The reaction was stopped by plunging the slides in a bath of 0.01 M PBS.
10. Washing	3 x 10 min in 0.01 M PBS
11. Mounting	Sections were mounted in 0.01 M PBS onto glass slides and left to dry overnight.
12. Counterstaining	10 min in 0.01 M PBS for hydration 40 s incubation in hematoxylin 1 min in in ddH_2O 5 min under running tap water
13. Dehydration	Baths of increasing ethanol concentrations. 1 min 70% ethanol 5 min 95% ethanol 10 min 100% ethanol
14. Embedding	2 x 5 min xylene Embedding in a xylene-based mounting medium Roti®Histokit, one drop per section. A cover glass should be firmly pressed on top of glass slide, carefully pushing away the air bubbles when necessary.

Figure 2. 3: Netwells™. 15mm Netwell™ Insert with 440 µm Mesh Size Polyester Membrane. Reproduced from http://www.corning.com

2.4.2.3 Antibodies

The following tables (2.16, 2.17) show the primary and secondary antibodies, their host organisms and corresponding dillutions.

Table 2. 16: Primary antibodies used for immunohistochemical stainings

Antibody	*Physiological target*	*Host*	*Dilution*	*Manufacturer*
Aβ [N]	Aβ1-x	Polyclonal Rabbit	1:500	IBL, Hamburg, Germany
Anti-Neurofilament-L #171002	Neurofilament-L	Monoclonal, Mouse	1:500	Synaptic Systems, Göttingen
DCX	Doublecortin, expressed by migrating neurons	Polyclonal, Goat	1:500	Santa Cruz Biotechnology, Inc., USA

Table 2. 17: Secondary antibodies applied for immunohistochemistry

Antibody	*Dilution*	*Manufacturer*
Rabbit anti-mouse immunoglobulins, biotinylated, E0465	1:200	DAKO, Denmark
Swine anti-rabbit immunoglobulins, biotinylated, E0353	1:200	DAKO, Denmark
Rabbit anti-goat immunoglobulins, biotinylated, E0466	1:250	DAKO, Denmark

2.5 Quantification of neuron number by stereology

2.5.1 Design-based stereology

Design-based stereology is an unbiased stereological method. Different quantities can be explored with stereology in order to make quantitative statements regarding function of tissues and organs. For example: the number of things such as number of brain cells within a brain region can be counted; length such as the length of axons can be measured; volume such as volume of the brain region; surface areas of the shape that is not flat such as the surface of a membrane can be estimated (West 2012).

Due to the stereology, it is possible to describe 3D structures from measurements that are made on 2D images (West 2012). Design-based stereology (further "Stereology") avoids all assumptions about the shape, size, spatial orientation or spatial distribution of the object of study, but uses carefully randomized probes (e.g. brain sections) for an unbiased estimation of different quantities. All sections are investigated with a uniform random probability. The results obtained with stereology can be easily reproduced. Another advantage of this method is efficiency. It allows estimating the number of cells of a brain region with a very low error margin using only few systematically sampled sections (Schmitz and Hof 2005; West 2002)

2.5.1.1 Optical dissector

The optical fractionator is the central tool of Stereology. It allows sampling a 3D region of interest and estimating the total number of particles (i.e. neurons in our case) (West et al. 1991). The optical fractionator method uses the optical dissector. The optical dissector is a 2D counting frame projected along the Z-axis.

The counting frame is usually shown as a red and green probe. Green and red lines are inclusion and exclusion lines, respectively (see figure 2.4). The third dimension is the *Z* dimension, the so-called disector height.

The positions of counting frames are determined by randomly placing a rectangular grid on the surface of a section (figure 2.5).

2.5.1.2 Counting rules and criteria for counting cells

During the counting, the set of rules must be followed in order to achieve unbiased estimation (Gundersen 1977; West et al. 1991). At first, the cell's unique point must be identified. In this study, the nucleus' top was used as the unique point. The unique point must fall within the disector height. The counting frame rules are applied when the unique point first comes into the focus. Cells should not be counted if the unique point comes into the focus while in the guard zone.

Guard zones are regions at the top and bottom of the tissue section in which cells must not be marked (figure 2.6). They are needed, because some cells can be damaged, cut or plucked from the tissue through the cutting prozess. Guard zones help to exclude them from counting.

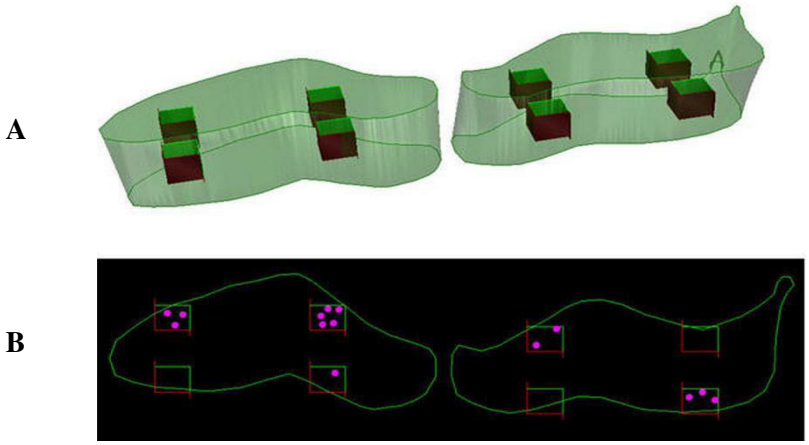

Figure 2. 4: The optical fractionator. The optical fractionator probe uses thick sections to estimate the total number of cells from the number of cells sampled (pink dots in (B)). (A)Three-dimensional representation of randomly placed 3D counting frames. (B) An optical disector is limited by the intersectional red and green lines. Reproduced from http://www.stereology.info/the-optical-fractionator/

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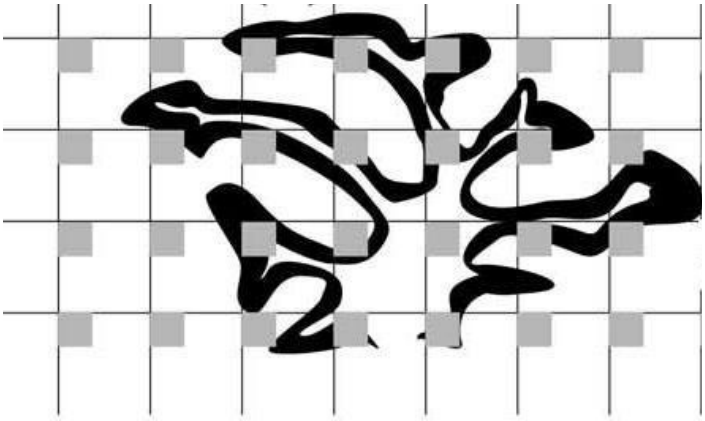


Figure 2. 5: Sampling grid. A randomly placed rectangular grid (big white squares) with side lengths *x* and *y* on the surface of a section, determining the positions of unbiased counting spaces (small grey squares). Figure modified from Schmitz and Hof 2005, p. 818.

A marker should be placed on the unique point, when the unique point first comes into focus. If any area of it touches the red exclusion line or touches/crosses both an inclusion line and an exclusion line, the cell must not be counted.

Figure 2.7 demonstrates how counting frame rules guarantee that cells are counted once and only once.

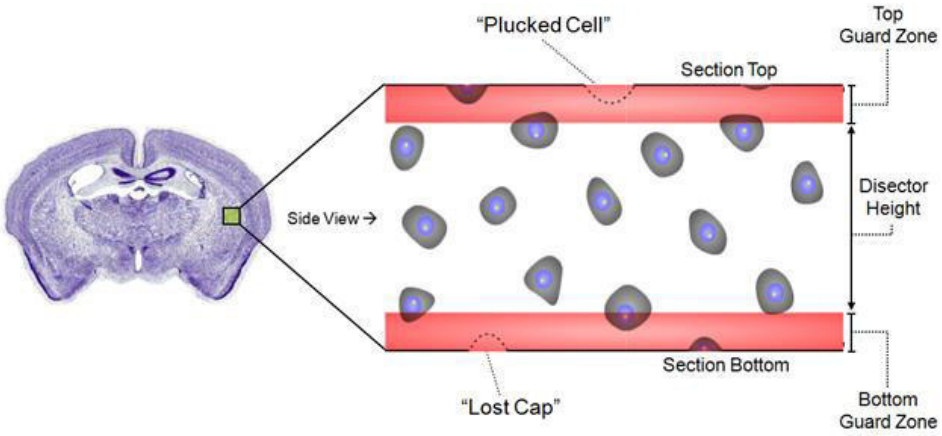


Figure 2. 6: Guard zones. Explaining the necessity to set them in order to have a homogenous sample. Reproduced from www.mbfbioscience.com

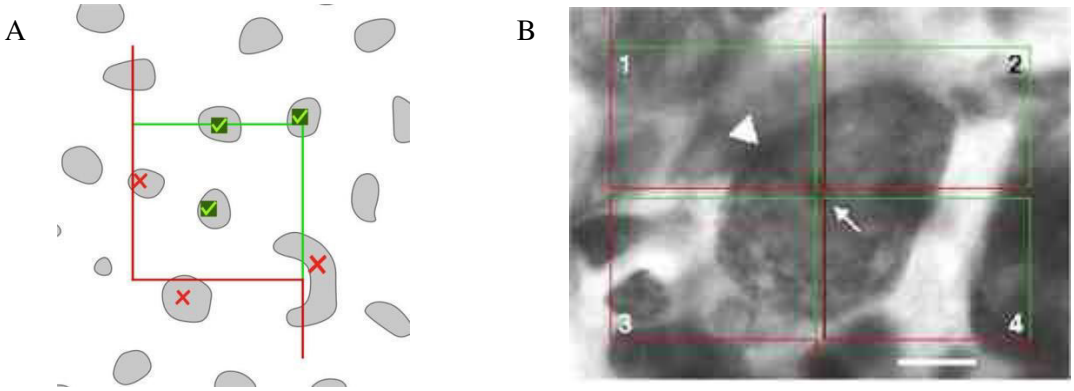


Figure 2. 7**:** Counting rules. (A) An example of using a counting frame to include or exclude objects: a cell is counted if it lies completely inside the counting frame or if it crosses a green line but not a red line. The grey shapes represent the unique points. (B) Four counting frames positioned very close to each other. The Purkinje cell appears in all counting frames, but it is only counted for frame 3, since it crosses the exclusion lines in frames 1, 2, and 4.
(A) and (B) are reproduced from http://www.stereology.info/criteria-for-counting-cells/ and http://www.stereology.info/counting-rules/ respectively.

2.5.2 Quantification of the total neuron number in the granular cell layer of the dentate gyrus

For the estimation of the total granule cell number in the dentate gyrus of Tg4-42$_{hom}$ mice, the design-based stereology (Schmitz and Hof 2005; West et al. 1991) (see 2.3.1) was applied.

The tissue for quantification was prepared as described in 2.3. The left hemispheres were cut frontally into entire series of 30-µm thick sections; every tenth section was systematically sampled and stained with cresyl violet (see 2.4.1).

The stereological analysis was performed as previously described (Casas et al. 2004; Schmitz et al. 2004)**.** The sampling grid area (*xy*), counting frame area (*XY*) and disector height (*Z*) were adapted to the evaluated region (granular cell layer of the dentate gyrus) (see table 2.18).

The granular cell layer of the dentate gyrus was delineated at low magnification (40X) on cresyl violet-stained sections (Bregma -1.34 to -3.80 (Paxinos and Franklin 2001)) (see figure 2.8). Using a stereology workstation equipped with an Olympus BX-51 microscope with a motorized specimen stage for automatic sampling (StereoInvestigator 7, Micro-BrightField, Williston, VT, USA), the neuronal nuclei were sampled at high magnification (1000X) as described in 2.5.1, and the total number of neurons was subsequently estimated by the fractional method using a 2-µm top guard zone. The section thickness was measured on every grid site with a dissector height (*Z*) of 5 µm. On average, 10 sections per animal were used to estimate the total neuron number of the granular cell layer of the dentate gyrus. In order to guarantee unbiased counting, all samples were blinded. The following numbers of animals were used in this analysis: Tg4-42$_{hom}$: n = 11 (6 m, EE), n = 8 (6 m, SH), WT: n = 3 (6 m, SH).

The number of cells in the region of interest was estimated by the following formula (Mouton 2002; Schmitz and Hof 2005; West et al. 1991, S. 199):

$$N = \sum_{i=1}^{n} Q_i^- \times \frac{1}{tsf} \times \frac{1}{asf} \times \frac{1}{ssf} \qquad (4)$$

Where:

- N: estimated total number of neurons in the dentate gyrus
- $\sum_{i=1}^{n} Q_i^-$: the sum of neuron numbers of all analysed sections in one animal, where Q_i are the total numbers of counted objects in each section i of one animal.
- *tsf*: thickness sampling fraction ($tsf = \frac{Z}{T}$, where Z and T are the disector height and the section thickness, respectively)
- *asf:* area sampling fraction ($asf = \frac{xy}{XY}$, where *xy* and *XY* are a sampling grid area and a counting frame area, respectively)
- *ssf*: section sampling fraction (in our case *ssf* = 10, because every tenth section was analysed.

The stereologic parameters' values used for the estimation of the DG are listed in the table 2.18.

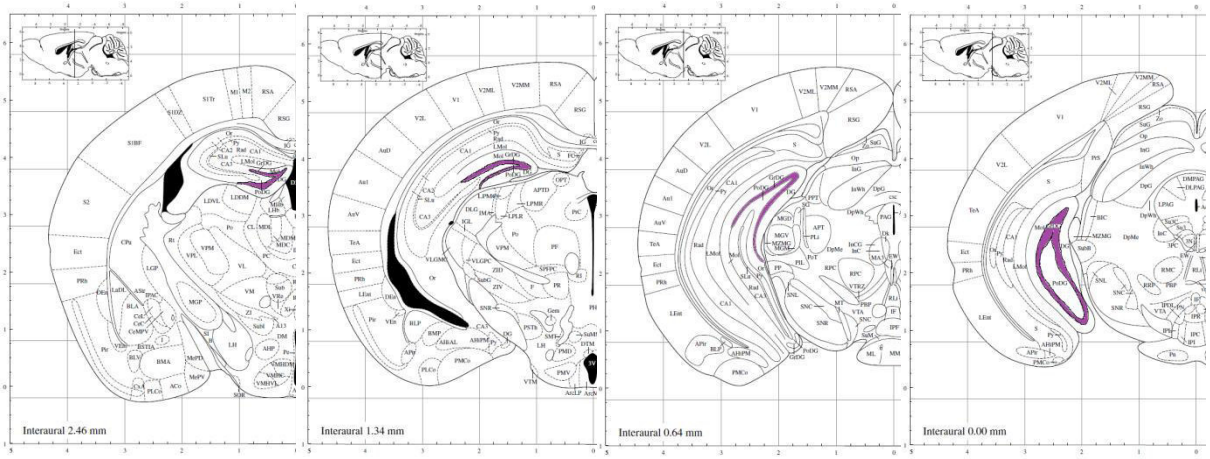


Figure 2. 8**:** Coronal slices of the mouse brain highlighting the dentate gyrus (adapted from Paxinos and Franklin 2001). DG is set off in violet ink. From left to right: Bregma −1.22, -2.46, -3.16 and −3.80 mm.

Table 2. 18: Parameters for stereological analysis of the granular cell layer of the dentate gyrus

Paramete	***DG***
Disector Height (Z) (µm)	5
Disector Volume (XYZ) (µm³)	980
Sampling Grid (x) (µm)	133
Sampling Grid (y) (µm)	75
Sampling Grid Area (xy) (µm²)	9975
Counting Frame Width (X) (µm)	14
Counting Frame Height (Y) (µm)	14
Counting Frame Area (XY) (µm²)	196
T (µm) [average value]	8.87
Z (µm)	5
tsf	0,6
asf	50.9
ssf	10

2.6 Estimation of the volume of the dentate gyrus

To estimate the volume of the dentate gyrus, Cavalieri's principle was used according to (Rosen and Harry 1990). Cavalieri's principle is an unbiased estimator, which is based on a rectangular approximation of the area under a curve and requires systematic sampling (Cruz-Orive 1985; Gundersen and Jensen 1987). This principle was claimed to be an efficient tool for volume estimation of irregular brain regions (Uylings et al. 1986)**.** Rosen and Harry (1990) confirmed this in their study. They suggested the use of Cavalieri's principle in all cases of volume estimation of regularly and irregularly shaped, equally spaced brain regions. When only a few sections are available, it provides the best estimate of the volume. Cavalieri's principle is demonstrated in the figure 2.9.

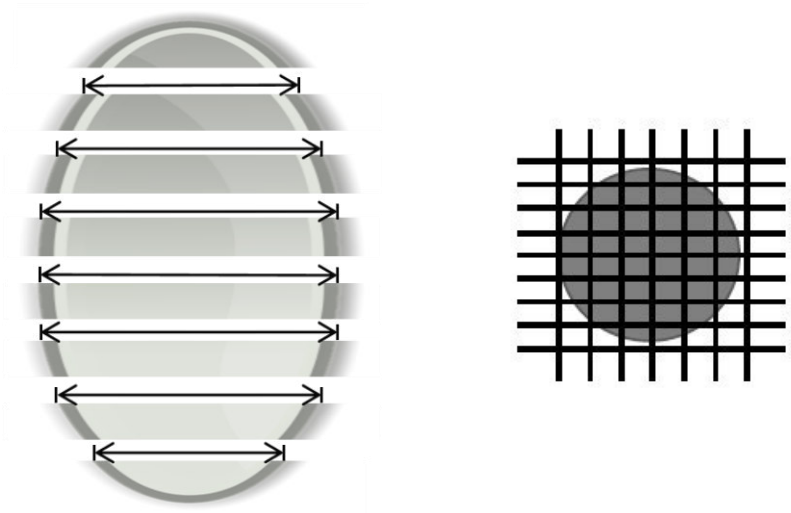
The mathematical formula used to calculate the volume is the following:

$$V_C = d \times \left(\sum_{i=1}^{n} y_i \right) - t \times y_{MAX} \quad (5)$$

Where:

- V_C: estimated volume
- d: distance between the sections that were analysed (in this case d = 300 µm as we analyse every tenth 30 µm-thick section)
- y_i: cross-sectional area of the i-th section through the morphometric region[4].
- n: total number of sections
- y_{MAX}: maximal value of y (maximum area)
- t: corresponding thickness of the y_{MAX} section (Gundersen and Jensen 1987)

The product of y_{MAX} and t is subtracted from the basic equation as a correction for overprojection. "Correction for overprojection is required when section thickness is not negligible, but the volume of the project is large" (Rosen and Harry 1990)



A **B**

Figure 2. 9: Cavalieri's principle for the estimation of the volume of a brain region. This principle is applied for volume estimation of regularly and irregularly shaped, equally spaced brain regions. (A) The brain region is cut into series of sections of uniform thickness, and the area of every x-th section is measured (arrows at the upper edge). An unbiased estimate of the volume of the brain region is obtained by multiplying the sum of its areas on all analysed sections with the uniform section thickness (Schmitz and Hof 2005). (B) The area of each section is measured by point counting method; in a square array test system the point area is calculated as the square of the distance between two points (Mandarim-de-Lacerda 2003, p. 475). The points are randomly but systematically distributed by a software program. Figure (A) created after Schmitz and Hof 2005, p. 819.

[4] Cross-sectional area of each section was estimated with StereoInvestigator 7 during the estimation of total neuron number of the dentate gyrus.

2.7 Quantification of Aβ plaque load

The extracellular Aβ load in the 5XFAD mice was evaluated in the cortex, the hippocampus, and the thalamus (figure 2.10) with an Olympus BX-51 microscope equipped with a Moticam Pro 282A camera (Motic) and the ImageJ software (V1.41, NIH, USA). Aβ[N], an antibody marking all Aβ species, was chosen for this analysis. The sagittal brain sections of 4 µm thickness were immunostained using DAB as a chromogene (see 2.4.2.1). Serial images of 100X or 200X magnification were captured on an average of four to six sections per animal, which were at least 30 µm afar from each other. Using ImageJ the pictures were binarized to 8-bit black and white images and a fixed intensity threshold was applied defining the DAB stained area. The area fraction covered by DAB staining was estimated.

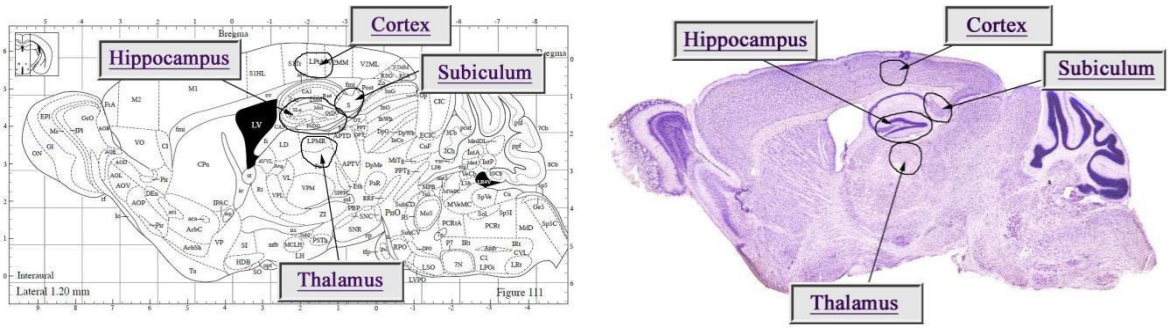


Figure 2. 10: Brain regions, in which the extracellular Aβ load was evaluated. Adapted from Paxinos and Franklin 2001.

For the statistical analysis, the mean values of the measurements in each animal were used. For the parts of the hippocampal formation such as the subiculum and the hippocampal region, ellipsoid frames of constant size adjusted to the desired brain region were created with ImageJ before quantification. The area outside of the ellipsoid was not included in the analysis (Figure 2.11). The protocol for Aβ plaque load quantification used in this study was elaborated by my colleague Bernhard Richard.

For better comparison, the relative Aβ load is expressed with $5XFAD_{het}$ mice as the reference parameter. The following number of animals was used in this analysis: $5XFAD_{hem}$: n=6 (2 m), n = 8 (5 m), n = 3–5 (9 m); $5XFAD_{hom}$: n=4 (2 m), n = 7 (5 m), n = 3-4 (9 m). One-way ANOVA followed by unpaired t-tests was used to compare age dependent changes in plaque load for each antibody (Richard et al. 2015).

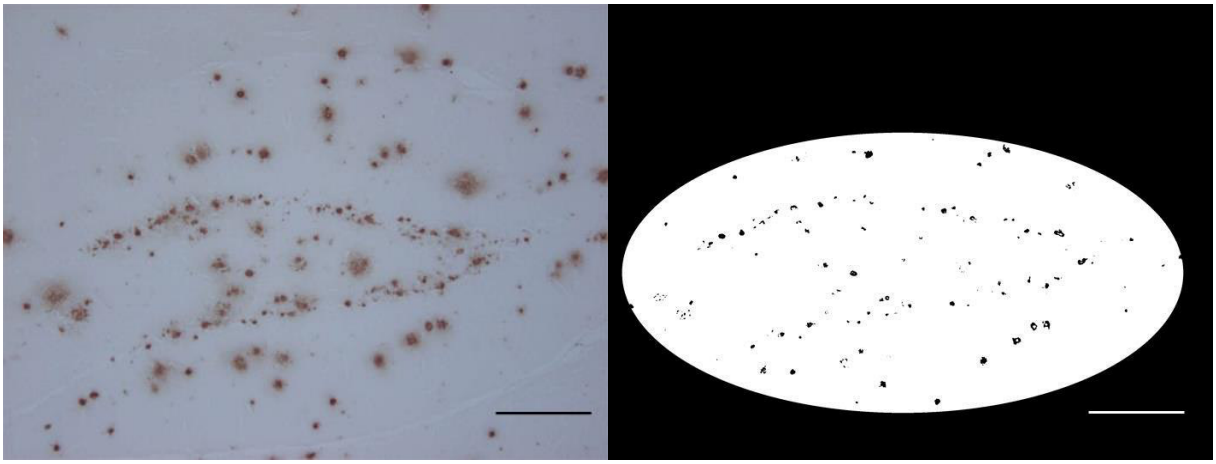


Figure 2. 11: An example of the application of the ellipsoid frame in the plaque load quantification. Hippocampus of a 5-month-old 5XFAD$_{hom}$ mouse. The initial image is on the left side; the image after application of the frame is on the right side. Scale bar: 200 µm. Source: own research.

2.8 Quantification of doublecortin-positive neurons and plaque independent neurofilament-positive axonal spheroids

The number of DCX-positive neurons within the subgranular zone (SGZ) of the dentate gyrus was quantified in order to estimate the adult neurogenesis in Tg4-42$_{hom}$ mice. The sections used for the estimation of adult neurogenesis were obtained by systematically collecting every tenth coronal frozen section of 30 µm-thickness (see 2.3 and 2.4.2.2). The number of DCX-positive neurons was estimated by multiplying the number of markers by a factor 10. The following number of animals was used in this analysis: Tg4-42$_{hom}$: n = 11 (6 m, EE), n = 8 (6 m, SH), WT: n = 5 (6 m, SH). The same Tg4-42$_{hom}$ mice were used for the estimation of the total neuron number of the dentate gyrus (see 2.5).

To estimate the axonal degeneration, paraffin sections of the brain and the spinal cord from 9-month-old animals were double-stained for neurofilament-L (see 2.4.2.1) and the number of the neurofilament-L-positive axonal spheroids was quantified. The estimation was performed on an average of four to six 4 µm-thick sections per animal which were at least 30 µm apart from each other (see 2.3). The following number of animals was used in this analysis: 5XFAD$_{hem}$: n = 3 (spinal cord), n = 5 (pons), 5XFAD$_{hom}$: n = 4 (both spinal cord and the pons). The dystrophic neurites that presented a diameter greater than 5 µm and that were not located in the immediate vicinity of amyloid plaques were counted as axonal spheroids.

The DCX-positiv neurons and neurofilament-L-positive axonal spheroids were counted using the Meander Scan platform of StereoInvestigator 7 (MBF Bioscience, Williston, VT, USA) at 1000X and 200X magnification.

2.9 Statistical analyses, software and images

The statistical analysis of the collected data was performed with GraphPad PRISM 5.01 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The differences between the groups were tested with a one-way analysis of variance (ANOVA) followed by unpaired t-tests. All data are presented as mean ± s.e.m. The significance levels were given as follows: $***p < 0.001$; $**p < 0.01$; $*p < 0.05$.

DNA in the gel after agarose gel electrophoresis was analysed with the Quantity One software program (Version 4.30; Biorad).

The qRT-PCR data were collected using the MxPro Mx3000P software (Stratagene, Santa Clara, CA, USA).

Stereo Investigator 7 software (MBF Bioscience, Williston, VT, USA) was used for design-based stereology.

The images of the stained tissue were acquired using a BX-51 microscope (Olympus, Shinjuku, Japan) equipped with with a Moticam Pro 282A camera (Motic).

The images for the plaque load quantification were processed with the ImageJ software (V1.41, NIH, USA).

Graphs were plotted with GraphPad PRISM. Figures were composed with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA).

2.10 Lists of chemicals, reagents, kits and technical devices

The following tables list the chemicals (table 2.19), reagents and kits (table 2.20) and technical devices (table 2.21), which were used in this study.

Table 2. 19: Chemicals

Chemical	***Manufacturer***
3,3'-Diaminobenzidin (DAB)	Roth, Karlsruhe, Germany
Acetic acid	Merck, Darmstadt, Germany
Agarose	Lonza, Basel, Switzerland
Boric acid	Sigma, St. Louis, MO, USA
Citric acid	Roth, Karlsruhe, Germany
Cresyl violet	Fluka, St. Louis, MO, USA
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
Formic acid 98%	Roth, Karlsruhe, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Hydrogen peroxide (H_2O_2)	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
Molecular grade water	Braun, Melsungen, Germany
Natriumacetate trihydrate	Roth, Karlsruhe, Germany
Paraformaldehyde (PFA)	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany
Sucrose	Roth, Karlsruhe, Germany
Tris (hydroxymethyl) aminomethane (Tris)	Roth, Karlsruhe, Germany
Triton X-100	Roth, Karlsruhe, Germany
Xylene	Roth, Karlsruhe, Germany

Table 2. 20: Reagents and kits

Reagent/Kit	***Manufacturer***
100 bp Ladder	Bioron, Ludwigshafen, Germany
10x reaction buffer	Axon, Kaiserslautern, Germany
Agarose Sample Buffer (Blue Juice)	Life Technologies, Carlsbad, CA, USA
Desoxyribonukleosidetriphosphates (dNTPs)	Axon, Kaiserslautern, Germany
Dulbecco's phosphate buffered salt solution (PBS)	PAN-Biotech, Aidenbach, Germany
DyNAmo Flash SYBR Green qPCR Kit	Thermo Fisher Scientific, Waltham, MA, USA
Fetal Calf Serum	Thermo Fisher Scientific, Waltham, MA, USA
Hematoxylin Solution	Roth, Karlsruhe, Germany
Ketamine 10 %	Medistar, Ascheberg, Germany

Reagent/Kit	***Manufacturer***
Magnesium chloride ($MgCl_2$) for PCR	Axon, Kaiserslautern, Germany
Non-Fat Dry Milk	Roth, Karlsruhe, Germany
Paraffin for tissue embedding	Roth, Karlsruhe, Germany
Primers for genotyping	Eurofins, Ebersberg, Germany
Proteinase K	Peqlab, Erlangen, Germany
Roti®Histokitt	Roth, Karlsruhe, Germany
Taq DNA-Polymerase	Axon, Kaiserslautern, Germany
Tissue-Tek®O.C.T.™ Compound (soluble glycol and resin compound)	SAKURA Finetek Europe
Vectastain Elite ABC Kit	Vestor Laboratories, Burlingame, CA, USA
Xylazine (Xylariem)	Ecuphar, N.V. Oostkamp, Belgium

Table 2. 21: Technical devices

Device	***Manufacturer***
Biophotometer	Eppendorf, Hamburg, Germany
Cenrifuge (Heraeus Biofuge Pico)	Thermo Fisher Scientific, Waltham, MA, USA
Centifuge (Heraeus Biofuge Stratos)	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge (Spectrafuge Mini)	Labnet Inc., Edison, NJ, USA
CM1850 UV Cryostat	Leica, Wetzlar, Germany
EG1140 H Embedding Station	Leica, Wetzlar, Germany
Electrophoresis chamber	Biorad Laboratories, Hercules, CA, USA
Embedding cassettes	Simport, Beloeil, QC, Canada
Eppendorf LoBind reaction tubes	Eppendorf, Hamburg, Germany
HM 335E Microtome	MICROM, Germany
Individually Ventilated Cages (IVC)	Tecniplast, Hohenpleissberg, Germany
LabCycler for PCR	SensoQuest, Goettingen, Germany
Microscope cover glasses	Thermo Fisher Scientific, Waltham, MA, USA
MX3000P Real-Time Cycler	Stratagene, Santa Clara, CA, USA
Netwells®	Corning Inc., Corning, NY
Odyssey FC	Li-Cor, Bad Homburg, Germany
Pap Pen Lipid Pen	Kisker Biotech, Steinfurt, Germany
PCR tubes	Greiner Bio-One, Kremsmünster, Austria
PCR tubes (for qRT-PCR)	Biozym, Oldendorf, Germany
Power Pack P25 power supply	Biometra, Goettingen, Germany
Superfrost® glass slides	Thermo Fisher Scientific, Waltham, MA, USA
Thermomixer Compact	Eppendorf, Hamburg, Germany
TP 1020 Automatic Tissue Processor	Leica, Wetzlar, Germany
Uvette® 220-1600 nm cuvette	Eppendorf, Hamburg, Germany
Vortexer (Vortex Genie 2)	Scientific Industries, Bohemia, NY, USA
Water bath for mounting of paraffin tissue	Medax, Olching, Germany

3. RESULTS

3.1 Project I: Physical activity ameliorates neuron loss and increases neurogenesis in 6-month-old Tg4-42$_{hom}$ mice.

3.1.1 Total neuron number: enriched environment and physical exercise attenuate neuron loss in Tg4-42$_{hom}$ mice.

The total neuron number of the granular cell layer of the dentate gyrus was quantified using design-based stereology in cresyl violet stained frozen sections of 6-month-old Tg4-42$_{hom}$ mice (Figure 3.1). The light microscopy revealed more lossened structure of DG in SH Tg4-42$_{hom}$ mice compared to the DG of EE Tg4-42$_{hom}$ mice. No shrinkage of DG was observed (Figure 3.1, A-D).

A statistically highly significant neuron loss ($p = 0.0002$) was observed in standard housed 6-month-old Tg4-42$_{hom}$ mice (446000 ± 24060, n= 8) compared to Tg4-42$_{hom}$ mice that lived under enriched conditions (591500 ± 18980, n = 11). The difference in the granular cell number between the SH and the EE group of mice was +25%. Stereological quantification showed similar numbers of neurons between WT mice (540000 ± 43140, *n* = 3) and EE Tg4-42$_{hom}$ mice (591500 ± 18980, n = 11). The difference in the cell numbers between SH Tg4-42$_{hom}$ and wild-type mice was -18% ($p = 0.0773$). There were highly significant differences between all three groups (one-way ANOVA, p = 0,0006). Therefore, stereology revealed that enriched hosing conditions ameliorate neuron loss in Tg4-42$_{hom}$ mice and restore the neuron number to the levels of the age-matched wild-types.

The numbers were estimated for the left hemispheres.

3.1.2 Volume of the dentate gyrus: no significant difference between EE Tg4-42$_{hom}$, SH Tg4-42$_{hom}$, and WT mice.

The volume of DG was estimated in order to analyze if physical activity and cognitive stimulation have an impact on it. Despite the massive neuron loss in the DG, the analysis of the volume of the dentate gyrus revealed no significant difference ($p > 0.05$) neither between EE Tg4-42$_{hom}$ and SH Tg4-42$_{hom}$ mice, nor between SH Tg4-42$_{hom}$ and wild-type mice (Figure 3.2).

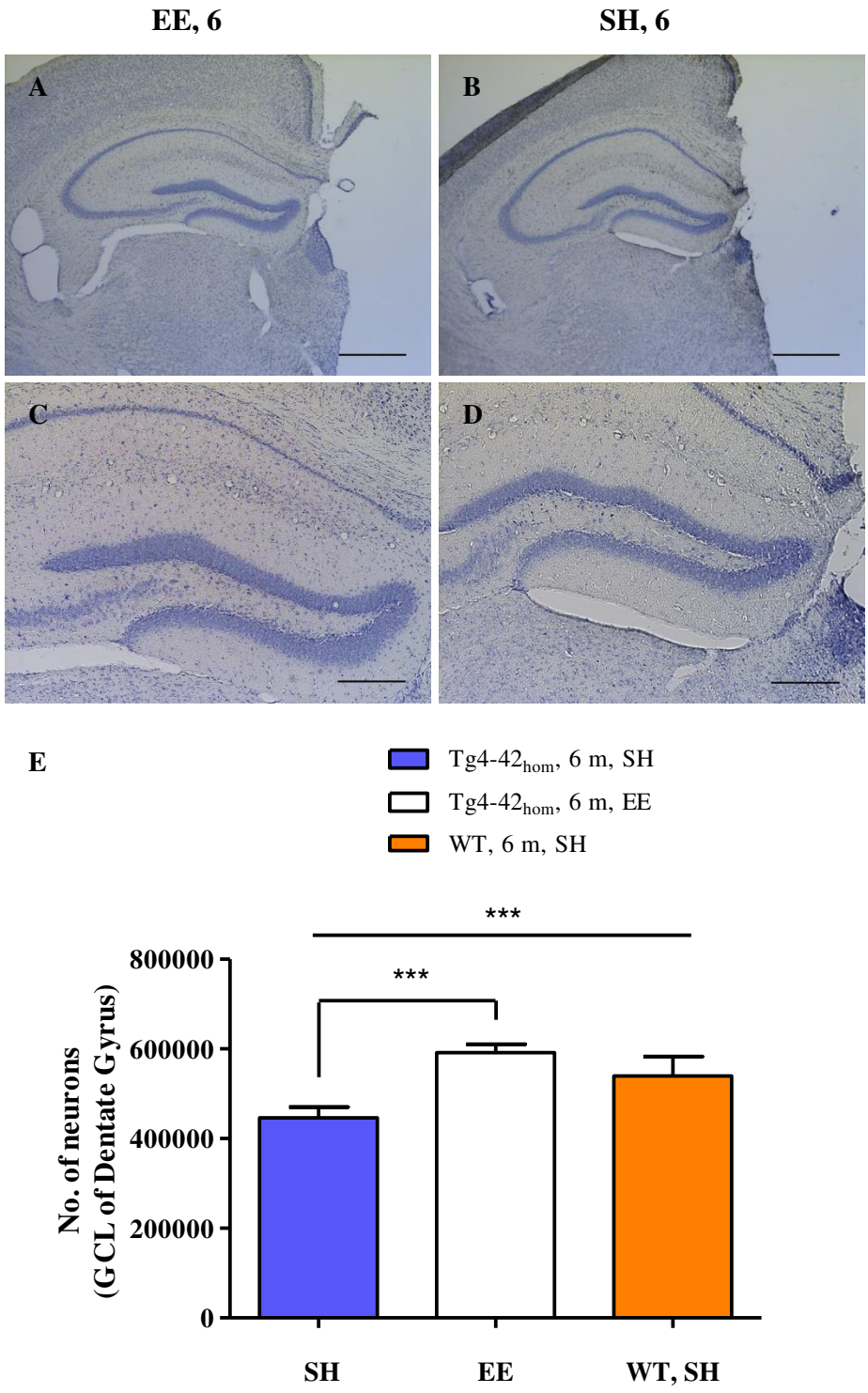


Figure 3. 1: Quantification of neurons in GCL of the dentate gyrus using unbiased stereology. (A-D) Cresyl violet stained coronal frozen sections (approx. Bregma -1.80 according to Paxinos and Franklin (2001)). Loosened structure of the DG in SH Tg4-42$_{hom}$ in comparison to EE Tg4-42$_{hom}$ mice. (E) Significant difference in the cell number between SH Tg4-42$_{hom}$ and EE Tg4-42$_{hom}$ mice. Data presented as mean ± s.e.m; ***$p < 0.001$; m = months. Scale bar (A-B): 500 µm; scale bar (C-D): 200 µm.

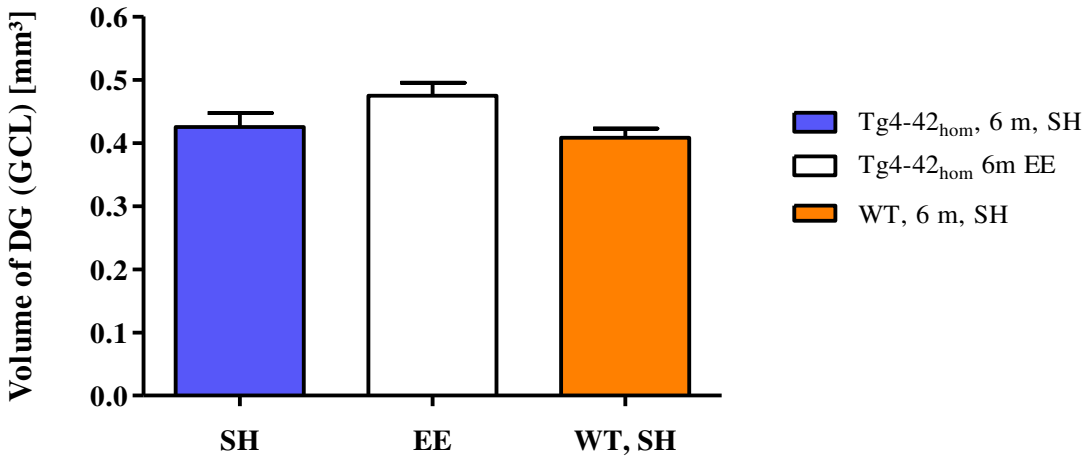


Figure 3. 2 Volume of the GCL of the dentate gyrus. No significant difference between SH Tg4-42$_{hom}$ and EE Tg4-42$_{hom}$ mice could be detected. Data presented as mean ± s.e.m; $p > 0.05$; m = months.

3.1.3 Effects of environmental enrichment on adult subgranular neurogenesis: enriched environment triggers neurogenesis in Tg4-42$_{hom}$ mice

In order to quantify the adult subgranular neurogenesis in Tg4-42$_{hom}$ mice and analyze if the enriched environment has an influence on it, free-floating frozen sections of Tg4-42$_{hom}$ mice brains were stained with an antibody against doublecortin (DCX). DCX is a microtubule-associated protein, which is exclusively expressed in immature neurons in the subventricular zone, where new neurons start migration to the olfactory bulbs, and in the subgranular zone of the dentate gyrus (Couillard-Despres et al. 2005; Lledo et al. 2006).

The analysis revealed a significant difference between Tg4-42$_{hom}$ mice that lived under enriched conditions with increased physical activity and environmental diversity (6920 ± 366.7; n = 11) and standard housed Tg4-42$_{hom}$ mice (4555 ± 506.9; n = 8). The neuron number was strongly increased (+35%) in EE-housed Tg4-42$_{hom}$ mice compared to SH Tg4-42$_{hom}$ mice (Figure 3.3, J). The number of new-born neurons in the SGZ of the DG of SH Tg4-42$_{hom}$ mice (4555 ± 506.9; n = 8) was dramatically reduced (-43%) in comparison with SH wild-types (7886 ± 359.9; n = 5). This decline could be restored by 4 months of housing under EE conditions.

Furthermore, DCX-positive neurons in DG of the standard housed Tg4-42$_{hom}$ mice showed atrophic, in the DCX-staining almost not visible dendrites in comparison with SH wild-types. The dendrites of DCX-positive cells of EE housed Tg4-42$_{hom}$ mice were also shorter and less branched than those of SH wild-types. However, they were more pronounced than the dendrites of standard housed Tg4-42$_{hom}$ mice (Figure 3.3, A-I).

The results of the Project I have been published in Hüttenrauch et al. 2016.

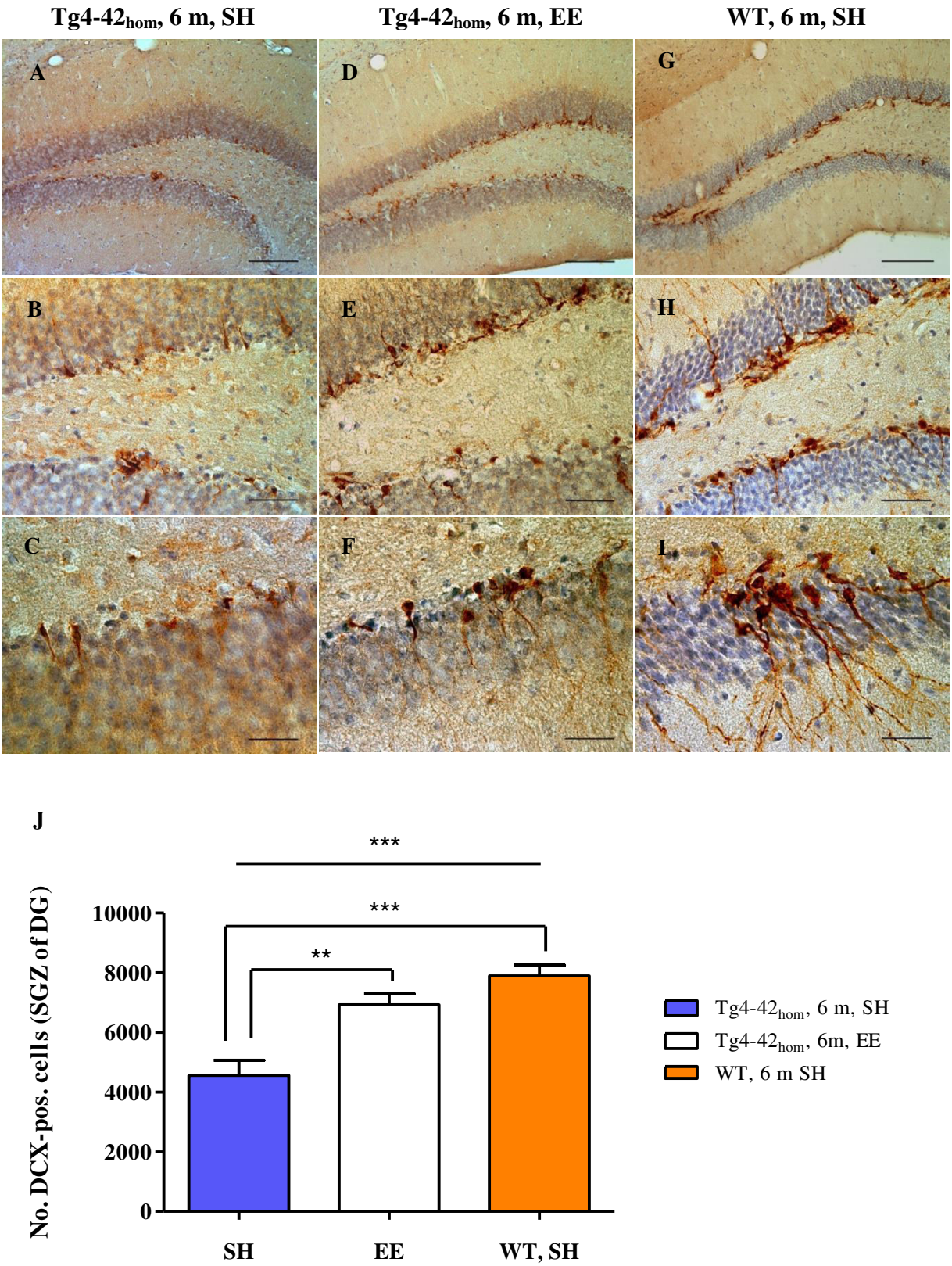


Figure 3. 3: Number of doublecortin-positive neurons in the SGZ of the dentate gyrus. (A-I) Coronal sections of Tg4-42$_{hom}$ and WT mice stained with antibody against DCX. (J) The neurogenesis was significantly increased (+35%) in EE Tg4-42$_{hom}$ mice in comparison to SH Tg4-42$_{hom}$ mice. The number of new-born neurons in the SGZ of the DG of SH Tg4-42$_{hom}$ mice was dramatically impaired (-43%) comparing with the WT mice. m = months. Data presented as mean ± s.e.m; ***$p < 0.001$, **$p < 0.01$. Scale bar (A, D, G): 100 µm; scale bar (B, E, H): 33 µm; scale bar (C, F, I): 20 µm.

3.2 Project II: Gene dosage-dependent increase of amyloid pathology and axonal degeneration in 5XFAD$_{hom}$ mice.

3.2.1 Quantification of Aβ plaque load

To quantify the gene dosage-dependent increase of the Aβ plaque load in 5XFAD mice, sagittal brain sections of 5XFAD$_{hem}$ and 5XFAD$_{hom}$ mice at 2, 5 and 9 months of age were stained with an Aβ[N] antibody, which detects an N-terminal Aβ epitope.

Relative amyloid load was quantified in the cortex, hippocampus (hippocampal region (CA and DG) (further "hippocampus"), retrohippocampal region (subiculum) (further "subiculum")) and the thalamus as described earlier (see 2.7) (Christensen et al. 2008). A significant increase in Aβ plaque load was found in 5XFAD$_{hom}$ compared to 5XFAD$_{hem}$ mice in all analysed brain regions at all time points (Cortex: 2 m: +232%; 5 m: +228%; 9 m: +32%; Hippocampus: 2 m: +615%; 5 m: +464%; 9 m: +52%; Subiculum: 2 m: +103%; 5 m: +66%; 9 m: 61%; Thalamus: 2 m: +311%; 5 m: +101%; 9 m: +75%) (Figure 3.6). Young mice at 2 months of age showed the highest increase. Aged mice at 9 months of age showed only moderate increases. The increase of the amyloid pathology is illustrated in the figures 3.4 and 3.5.

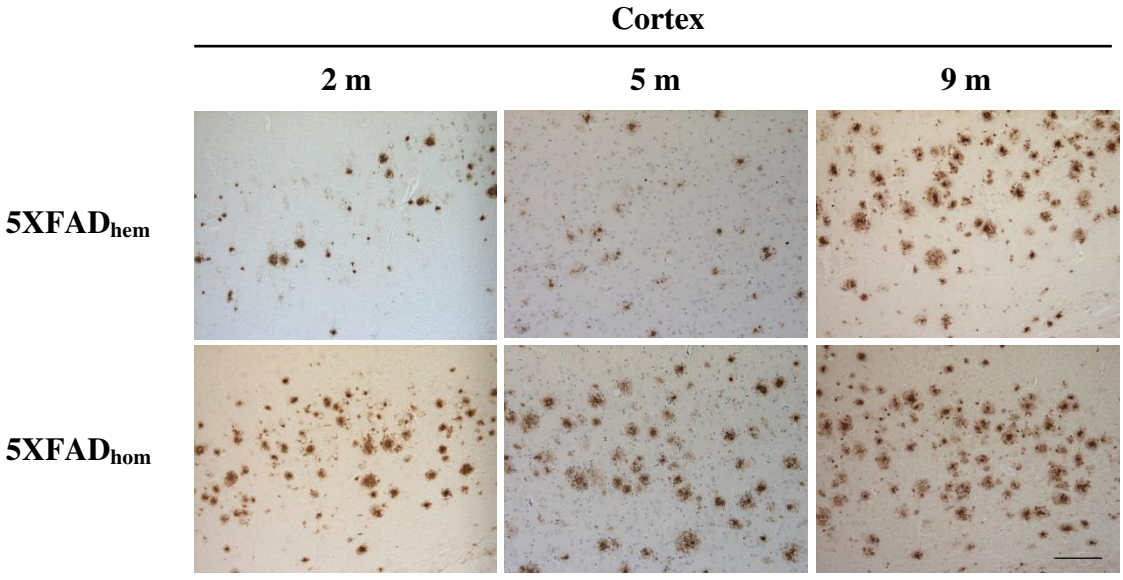


Figure 3. 4: Illustration of extracellular Aβ plaque load in cortex. Strong increase of amyloid load in 5XFAD$_{hom}$ mice at all time points compared to 5XFAD$_{hem}$. Scale bar: 100 µm.

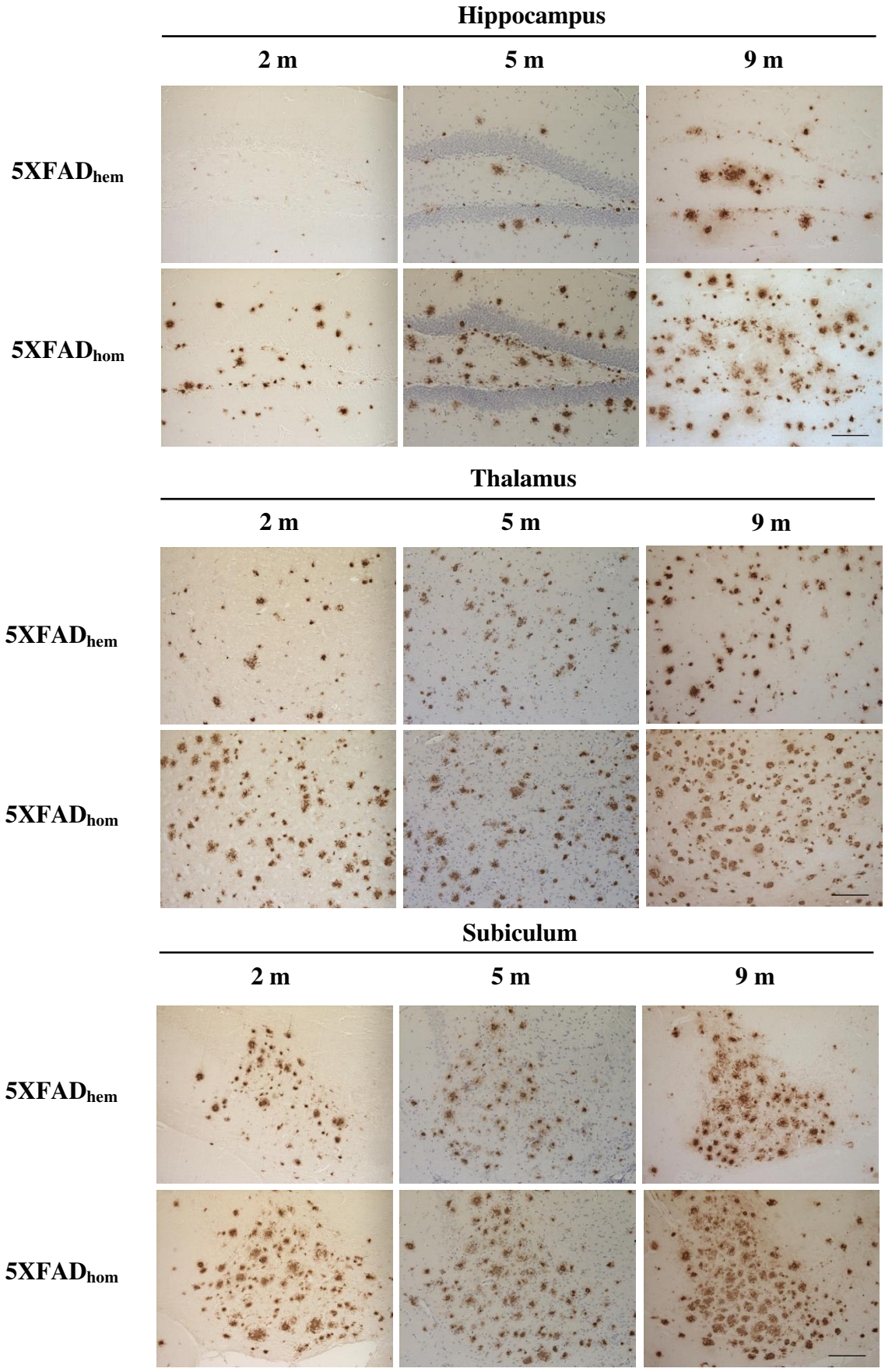


Figure 3. 5: Illustration of extracellular Aβ plaque load in hippocampus, subiculum and thalamus. Strong increase of amyloid load in $5XFAD_{hom}$ mice in all analysed brain areas at all time points compared to $5XFAD_{hem}$. Scale bar: 100 µm.

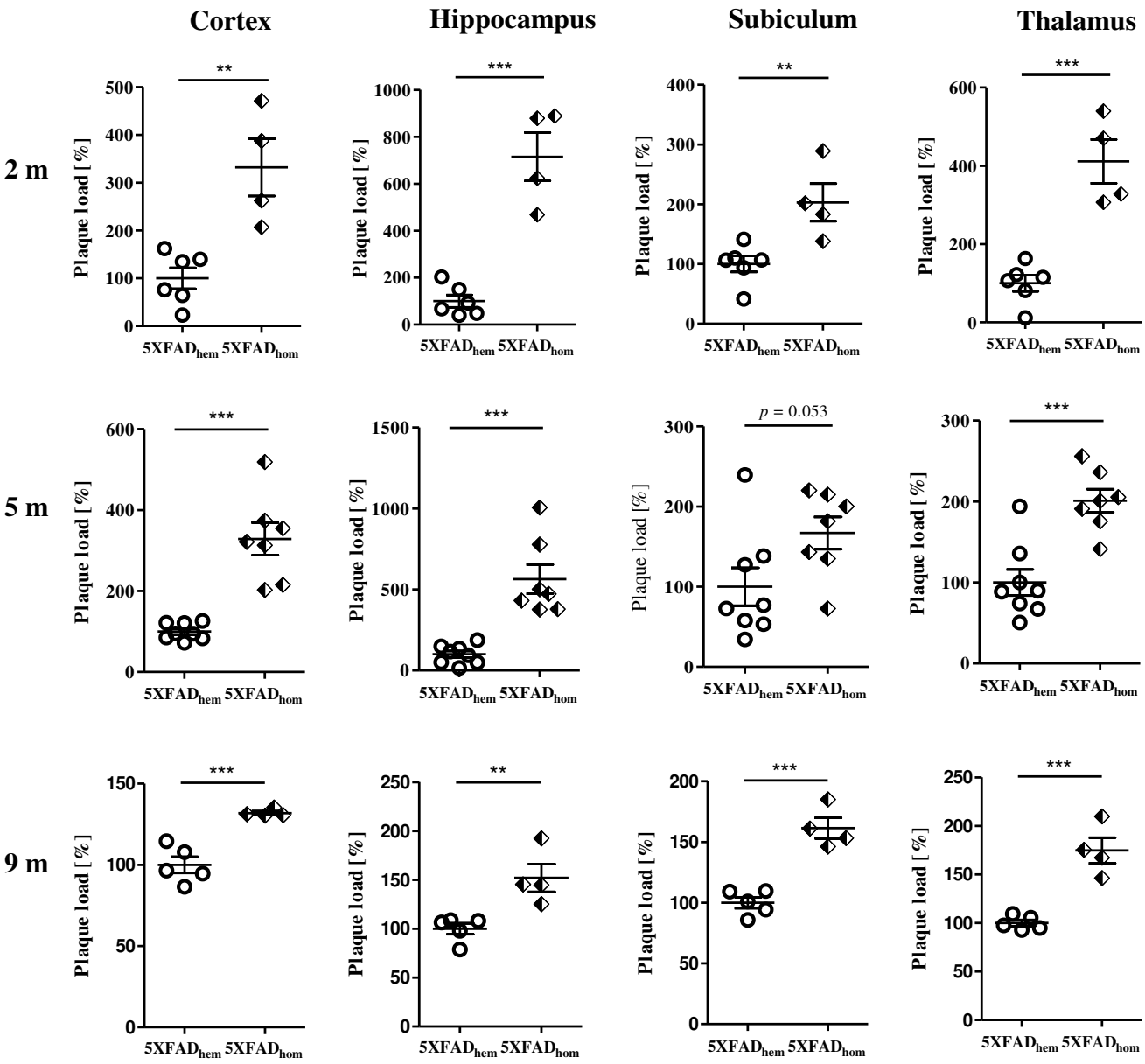


Figure 3. 6: Quantification of extracellular Aβ plaque load in cortex, hippocampus, subiculum and thalamus. 5XFAD$_{hom}$ mice showed a strongly increased plaque load in all analysed brain areas at all time points compared to age-matched hemizygous 5XFAD. For better comparison, the relative Aβ load is expressed with 5XFADhet mice as the reference parameter. m = months. Data presented as mean ± s.e.m; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

3.2.2 Quantification of neurofilament-positive axonal spheroids

The sagittal brain sections of 5XFAD$_{hem}$ and 5XFAD$_{hom}$ mice of 9 months of age were stained against neurofilament-L (a neurofilament subunit) in order to evaluate the gene dosage-dependent effect on the extent of axonal pathology. Neurofilament-positive axonal swellings (spheroids) that were not located in the immediate vicinity of amyloid plaques were counted in the pons and spinal cord.

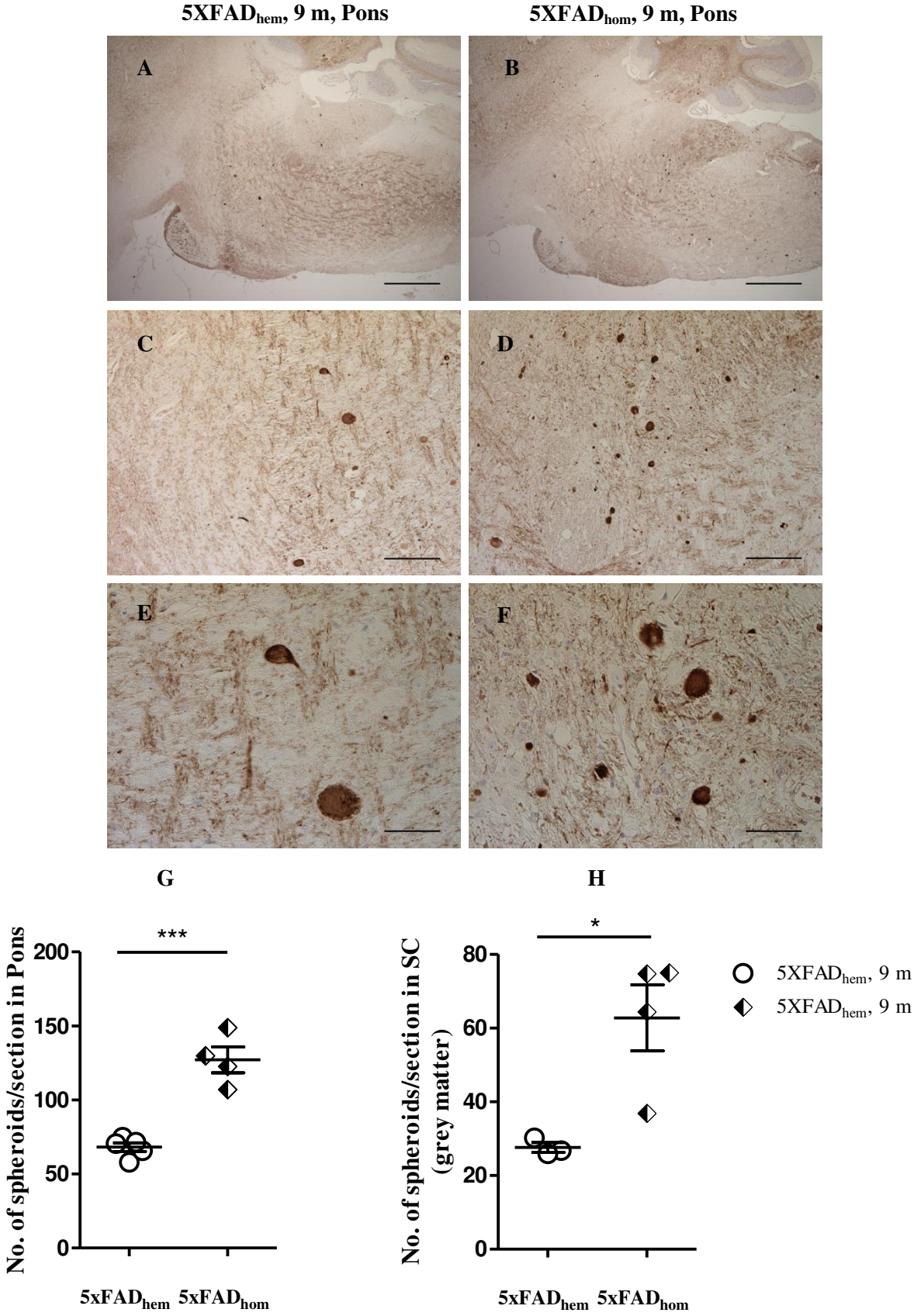


Figure 3. 7: Quantification of neurofilament-positive axonal spheroids in the pons and spinal cord. (A-F) Neurofilament-L stained brain sections; scale bar (A-B): 500 µm; scale bar (C-D): 100 µm; scale bar (E-F): 33 µm. (G-H) The 9-month-old 5XFAD$_{hom}$ mice revealed a significantly increased number of axonal swellings in both regions compared to 5XFAD$_{hem}$ mice. m = months. Data presented as mean ± s.e.m; ***$p < 0.001$, *$p < 0.05$.

The number of spheroids per section in the pons in $5XFAD_{hom}$ mice was significantly increased (127.1±17.35; *n* = 4) compared to age-matched $5XFAD_{hem}$ mice (67.11±6.27; n = 5; p = 0.0002). The same was valid for the gray matter of the spinal cord. The number of axonal swellings in $5XFAD_{hom}$ was also strongly increased (62.74 ± 17.93; n = 4) compared to age-matched $5XFAD_{hem}$ mice (27.64 ± 2.27; n = 3) (Figure 3.7).

The results of the Project II have been published in Richard et al. 2015.

4. DISCUSSION

Mouse models are very important tools in the AD research that help us to understand the pathogenesis of AD, prevent it or develop treatments against it. In the present study two mouse models of AD have been investigated. A novel model Tg4-42, which is a model for sporadic AD, and a well-established 5XFAD model for familial AD were the subjects of this work.

The first project aimed to quantify the neuron loss and neurogenesis in the DG of 6-month-old Tg4-42$_{hom}$ mice and to determine the effects of environmental enrichment, in particular physical activity, on these histopathological features. In the second project, the 5XFAD model was investigated in terms of amyloidosis and axonal impairments under the influence of the gene dosage elevation.

In the following part of this work I would like to discuss the results of the present study.

4.1 Project I: Physical activity ameliorates neuron loss and increases neurogenesis in 6-month-old Tg4-42$_{hom}$ mice.

Tg4-42 is a novel model for sporadic AD created by our group, which expresses exclusively N-truncated Aβ_{4-42}. Aβ_{4-42} belongs to the major Aβ isoforms in the AD brain and its expression induces an age-dependent CA1 neuron loss associated with a severe memory decline (Bouter et al. 2013a).

As already mentioned, the aim of this study was to further characterize the Tg4-42 mouse model, investigate neuron loss in further hippocampal region such as dentate gyrus, and find out, whether physical, social and sensorial stimulation can prevent neuron loss and rescue neurogenesis in the dentate gyrus in Tg4-42 model. At the same time other colleagues in our group investigated, whether enriched living conditions can counteract CA1 neuron loss and ameliorate behavioural deficits in Tg4-42 mice (Hüttenrauch et al. 2016). The EE paradigm was used for both studies. The EE paradigm consists of a combination of voluntary exercise and cognitive stimulation. In the present study, homozygous Tg4-42 mice were housed under EE conditions. The EE housing was started before disease onset and continued until 6 months of age (the time point were SH mice display severe behavioural deficits and serious loss of CA1 hippocampal neurons).

In this study, unbiased design-based stereology revealed that EE ameliorates neuron loss in homozygous Tg4-42 mice. The number of DG cells in 6-month-old Tg4-42$_{hom}$ mice was restored to the levels of the wild type mice. In Hüttenrauch et al. (2016), our group also reported that robust deficits in spatial reference memory, impaired non-spatial learning and memory were rescued by EE housing conditions.

There are plenty of studies showing that increased cognitive activity and leisure-time physical activity during the life are associated with decreased risk of dementia and AD in old age (Rovio et al. 2005). It has been shown that enriched living conditions and especially physical activity have beneficial effect on cognitive performance (Kempermann et al. 1997; van Praag et al. 1999). With regard to this, EE paradigm was intensely investigated in terms of rescuing memory and learning abilities in animal models of neurodegenerative diseases (Nithianantharajah and Hannan 2006). Some results from human (Hamer and Chida 2009; Rolland et al. 2008) and animal studies (Görtz et al. 2008; Nichol et al. 2007) showed that physical activity and cognitive stimulation can prevent or slow down the AD progression. Many other reports on enriched environment also showed improvement in cognitive tests (Arendash et al. 2004; Costa et al. 2007; Jankowsky et al. 2005).

However, to the best of my current knowledge, no study was conducted, which shows the positive effects of environmental enrichment on the prevention of neuronal loss in AD mouse models so far. Our group previously published an EE study on APP/PS1KI mice, demonstrating that the housing condition had no influence on the selective loss of hippocampal CA1 neurons (Casas et al. 2004; Cotel et al. 2012). In contrast, the present study indeed shows a beneficial effect of physical activity on DG neuronal loss in an AD mouse model. An explanation for these at first glance conflicting results might be that the APP/PS1KI mouse model is a very robust and aggressive model of familial AD with several mutations, the effects of which cannot be ameliorated by rather mild interventions such as increased physical activity, cognitive and social stimulation. On the contrary, the Tg4-42 mouse model only expresses A$\beta_{4\text{-}42}$ and does not possess any mutations, and therefore rather represent a model of sporadic AD which might be able to be modified through environmental interventions like physical activity.

Some other AD mouse models upon enriched environment (TgCRND8, APP/PS1ΔE9) show a decreased amyloid plaque load (Adlard et al. 2005; Lazarov et al. 2005). Tg4-42 mice do not develop any extracellular amyloid plaques. They only secrete A$\beta_{4\text{-}42}$ soluble neurotoxic aggregates, predominantly in the hippocampus (Bouter et al. 2013a). It would be legitimate to assume that Tg4-42 mice would also display at least decreased levels of A$\beta_{4\text{-}42}$. Strikingly, improved cognitive performance and reduced neuron loss in Tg4-42$_{het}$ mice housed under EE was not accompanied with decreased levels of A$\beta_{4\text{-}42}$ (Hüttenrauch et al. 2016). Hence, it can be hypothesized that the cognitive improvement and the neuroprotective effect induced by physical activity in Tg4-42 mice is not dependent to a simple reduction of A$\beta_{4\text{-}42}$ levels. Similar effects were observed in other mice models e.g. Tg2576 or APP23 demonstrating improved cognitive performance despite unchanged Aβ levels (Arendash et al. 2004) or amyloid plaque load (Wolf et al. 2006). Therefore, in this case, it can be speculated that the beneficial effects of environmental interventions can be attributed to the expansion of cognitive reserve that helps coping with pathological changes in brain during the AD (Akbaraly et al. 2009; Albert et al. 1995).

Interestingly, the quantitative analysis of the volume of the DG revealed no significant difference between three groups. The same was also observed in the APP/PS1KI mice, where the volume of DG also remained constant despite the loss of DG cells (Cotel et al. 2012). Microscopically, the DG of SH Tg4-42$_{hom}$ mice showed loosened structure without shrinkage in comparison to the DG of EE Tg4-42$_{hom}$ mice.

As already stated, neuron loss and cortical atrophy are very important neuropathological hallmarks in AD patients. However, not all transgenic models of AD do show substantial neuron loss. Neuronal loss has not been reported for some APP transgenic models such as PDAPP (Games et al. 1995), Tg2576 (Hsiao et al. 1996), and APP/PS1ΔE9 (Borchelt et al. 1997). Mainly mouse models with multiple APP and PSEN-1 mutations display this cardinal feature of AD. For instance, 5XFAD mice show significant neuron loss in the fifth cortical layer of the frontal cortex from the 9 month of age. Similar to 5XFAD mice, substantial neuron loss is observed in the frontal cortex and CA1 region of hippocampus in APP/PS1K1 mice (Breyhan et al. 2009; Christensen et al. 2008; Eimer and Vassar 2013; Jawhar et al. 2012).

Interestingly, despite almost ubiquitous appearance of plaques in these mouse models, neuron loss occurs only in the regions with intraneuronal Aβ accumulation. This observation speaks more likely against the toxic role of extracellular plaques and supports the theory about toxicity of intracellular Aβ accumulation. In Tg4-42 mice, the dramatic progressive neuron loss also occurs in the regions of intracellular Aβ$_{4-42}$ accumulation. In the Tg4-42 mice, long-term exposure to N-truncated Aβ$_{4-42}$ induces an age- and dose-dependent neuron loss in CA1, which obviously contributes to the learning and memory deficits (Bouter et al. 2013a). These findings support the theory that intraneuronal Aβ, in particular Aβ$_{4-42}$, plays a crucial role in the process of neurodegeneration. The fact that Tg4-42 mice do not develop extracellular amyloid plaques but still demonstrate the majority of the cardinal AD features also speaks in favour of intracellular amyloid hypothesis (Bouter et al. 2013a).

While studying the impact of environmental enrichment, in particular physical activity, one of the main issues is the neurogenic effect of the EE paradigm. Increased hippocampal neurogenesis is one of the major effects that have been observed in the adult brains of rodents (Kempermann et al. 1997; Kempermann et al. 1998a; Kempermann et al. 1998b; van Praag et al. 1999). In this study, the analysis of doublecortin-positive new-born neurons in the DG of the standard housed Tg4-42$_{hom}$ mice revealed a very low amount of doublecortin-positive cells with atrophic, in the DCX-staining not visible dendrites in comparison with SH wild-types. On the contrary, the EE-housed Tg4-42$_{hom}$ mice showed strongly increased number of DCX-positive cells comparing to age-matched standard housed Tg4-42$_{hom}$ mice. Housing under EE conditions was able to restore the number of new-born neurons almost to the levels of SH wild-types. Qualitative analysis of the dendrites of DCX-positive cells on the basis of microscopy revealed also a stronger dendritic branching in EE-housed Tg4-42$_{hom}$ mice comparing to the standard-housed Tg4-42$_{hom}$ group. Nevertheless, SH Tg4-42$_{hom}$ mice hadless pronounced dendrites (shorter and less branched) in comparison to the wild-type aged-matched SH-mice. These findings are in line with with previous reports claiming that running stimulates the dendritic arbor of newborn cells in the

DG (Dostes et al. 2016; Marlatt et al. 2013). The explanation for restored total numbers of DG-cells in EE Tg4-42$_{hom}$ mice could be the rescued neurogenesis that leads to replacement of dead cells. Decreased hippocampal neurogenesis has been previously described also in other mouse models of AD such as PDAPP (Donovan et al. 2006), TgCRND8 (Herring et al. 2009), and 3xTg mice (Rodríguez et al. 2008).

In conclusion, the present study is the first to demonstrate that long-term physical activity has a preventive effect on Aβ-induced neuron loss and impaired neurogenesis in a transgenic mouse model of sporadic AD. Our results support the data obtained in retrospective epidemiological studies on human AD. Translating the findings of this animal study to the human AD patients, it can be assumed that physical activity and enriched environment may be beneficial in patients with mild cognitive impairment (before obvious disease onset). However, there are still a lot of questions remained unanswered. What is the reason of impaired neurogenesis in the SGZ of the DG? Is it due to the direct influence of Aβ$_{4-42}$ or due to the possible inflammatory climate (Verret et al. 2007)? Some studies showed a strong negative correlation between the number of surviving new born neurons and the number of activated microglia cells (Ekdahl et al. 2003) and described new-born neurons as highly vulnerable to inflammation. Does the neurogenic dysfunction originate from impaired cell cycle or from impaired differentiation or migration of new-born cells (Zitnik and Martin 2002)? Are physical activity and cognitive stimulation reliable enough for rescuing dying neurons? Further investigations need to be done also in order to clarify whether a later exposure to physical activity and environmental enrichment, i.e. after the onset of cognitive and behavioural deficits, would reduce the gravity of these symptoms and would still ameliorate neuron loss and neurogenesis.

Unfortunately for AD research, rodents do not develop AD spontaneously during the aging process (Sarasa and Pesini 2009). The transgenic mouse models of AD based on mutations in AD-related genes are relevant, in the first line, for the investigation of familial AD, which makes up barely 1 % of AD cases (Zetterberg and Mattsson 2014). The majority of cases are caused by sporadic AD. Regarding to this fact, the translation of findings in animal studies to the situation of sporadic AD patients is complicated. Thereby, the Tg4-42 mouse model, which reflects the sporadic form of the disease, is an important tool in the investigation of sporadic AD.

4.2 Project II: Gene dosage-dependent increase of amyloid pathology and axonal degeneration in 5XFAD$_{hom}$ mice

The 5XFAD mouse model is a double transgenic AβPP/PS1 mouse line co-expressing five familial AD mutations that are inherited together. This leads to massive and early plaque formation, intraneuronal Aβ aggregation, elevated Aβ$_{42}$ levels, behavioural deficits and neuron loss in the cortical layer V. 5XFAD was previously described as one of the few

AD models with several cardinal features of AD including neuron loss (Jawhar et al. 2012; Eimer and Vassar 2013; Oakley et al. 2006).

The present study aimed at performing an analysis of a potential gene dosage-dependent effect on neuropathological features in 5XFAD mice such as plaque load and axonopathy. In order to elevate the transgene expression and the Aβ levels, a homozygous 5XFAD line was created (Richard et al. 2015). Breeding transgenic mouse models of AD to homozygosity is an established method to aggravate the extent and accelerate the onset of pathological alterations. Increasing the gene dosage has been previously performed in other mouse models of AD such as PDAPP (German et al. 2003), ARTE10 (Willuweit et al. 2009) and Tg4-42 (Bouter et al. 2013a).

In this study, it has been shown that the levels of extracellularly deposited amyloid are significantly elevated in the $5XFAD_{hom}$ groups compared to the respective $5XFAD_{hem}$ groups in all analysed brain regions at all time points. In particular significant increase in Aβ plaque load was found in the cortex, the hippocampus, the subiculum and the thalamus. This was most evident at the earlier stages (2-month-old and 5-month-old mice) with 3- to 7-fold increases. Interestingly, 9-month-old mice showed only moderate increase in Aβ plaque load. This might reflect a saturation effect. This finding is consistent with the initial description of 5XFAD, which although not quantified, indicated a slower accumulation of Aβ between 6 and 9 months of age (Oakley et al. 2006). A similar plateau stage has also been observed in the APP/PS1KI and in the PDAPP model (DeMattos et al. 2012; Wirths et al. 2010c).

In the present study, besides the aggravation of the amyloid plaque pathology, it was demonstrated that $5XFAD_{hom}$ mice at 9 months of age show severe axonal degeneration in the pons and the spinal cord compared to age-matched heterozygous mice. The immunohistochemistry on brain and spinal cord with anti-neurofilament-L antibody revealed a significantly increased number of axonal swellings per section in $5XFAD_{hom}$ compared to $5XFAD_{hem}$ mice. This correlates positively with the earlier onset of motor deficits (Richard et al. 2015). An age-dependent axonopathy in brain and spinal cord of 5XFAD has been previously described (Jawhar et al. 2012). Spinal cord pathology including formation of dystrophic neurites, amyloid plaques, and motor neuron degeneration has been also described in several other mouse models overexpressing mutant APP (Christensen et al. 2014; Seo et al. 2010; Wirths et al. 2006; Wirths et al. 2007; Yuan et al. 2013). Furthermore, intracellular Aβ accumulation in spinal cord motor neurons has been observed in several APP transgenic e.g. APP/PS1 (Wirths et al. 2006) or APP/PS1KI (Christensen et al. 2014; Wirths et al. 2007), as well as in mice expressing only Aβ peptides without APP overexpression such as APP48 mice expressing $Aβ_{1-42}$ (Abramowski et al. 2012) or TBA42 mice expressing $Aβ_{pE3-42}$ (Wittnam et al. 2012). Several studies suggested that fibrillary Aβ leads to the formation of axonal swellings (Grace et al. 2002; Selkoe 2002). This subsequently causes local accumulation of cell components such as vesicles, mitochondria, vacuoles, that leads to the starvation of synapses and dying-back neurons.

Axonal damage analogous to axonopathy in 5XFAD mice has been also reported in AD patients. For example, signs of axonopathy and decreased axonal transport rate have

been shown in vivo by MRI[5] (Minoshima and Cross 2008) as well as in post mortem studies (Dai et al. 2002; Xiao et al. 2011). Whereas there is a plethora of literature on cognitive decline during the course of AD, detailed reports on motor impairments in AD are limited (Albers et al. 2015; Wirths and Bayer 2008). Impaired motor performance has been reported in later disease stages (Albers et al. 2015; Cummings 1990), but there is an evidence that they are also present in mildly demented AD patients (Goldman et al. 1999). Some studies suggested that motor deficits even might be a characteristic feature of early AD pathology (Scarmeas et al. 2004) and bear some predictive value (Scarmeas et al. 2005).

Other gene dosage-dependent effects in the 5XFAD model

In Richard et al. (2015), along with the described acceleration of amyloid plaque deposition and significantly increased axonal degeneration, we have shown that the development of behavioural impairment was also accelerated and the levels of both TBS- and SDS-soluble Aβ peptides were significantly elevated in the $5XFAD_{hom}$ groups compared to the respective $5XFAD_{hem}$ groups. Behavioural experiments, phenotypical characterisation of the mice and quantification of TBS- and SDS-soluble Aβ peptides were performed by my colleague Bernhard Richard.

The onset of behaviour deficits was considerably shifted towards earlier time points in the $5XFAD_{hom}$ strain. For example, a robust significant impairment of the spatial reference memory was observed already at the age of 5 months for the $5XFAD_{hom}$ group, whereas age-matched $5XFAD_{hem}$ did not show any impairment in corresponding tasks. Due to homozygosity, not only the onset of behavioural impairment was shifted toward earlier time points. It was accompanied by an aggravation of other alterations such as reduced body weight and faster development of a motor phenotype for $5XFAD_{hom}$ (Richard et al. 2015).

For the quantification of soluble Aβ peptides whole-brain lysates (TBS and SDS-fraction) of 2- and 5-month-old animals were subjected to quantitative ELISA measurement. ELISA measurements were performed by Sandra Baches, Dept. of Neuropathology, Heinrich Heine Universität Düsseldorf, Germany. Water-soluble $Aβ_{1-42}$ levels, as well as SDS-soluble $Aβ_{1-40}$ and $Aβ_{1-42}$ were significantly elevated in $5XFAD_{hom}$ animals (2 and 5 months of age) compared to age-matched $5XFAD_{hem}$ (Richard et al. 2015). These findings are consistent with the increased plaque load observed in $5XFAD_{hom}$ mice.

It cannot be discriminated whether extracellular amyloid depositions or soluble Aβ are responsible for behavioural deficits. There is evidence that amyloid plaques do have neurotoxic effects, especially if Aβ depositions adopt a fibrillary β-pleated conformation (Urbanc et al. 2002). However, it has been also shown that mice without amyloid plaque accumulation but considerable levels of soluble Aβ peptides demonstrate an altered synaptic structure (Price et al. 2014).

In summary, homozygous 5XFAD mice demonstrate abundant Aβ pathology, a robust behavioural phenotype with deficits in reference learning and memory, and pronounced

[5] Magnetic Resonance Imaging

axonopathy. Increasing the transgene dose in 5XFAD leads to increased transgene expression and elevated Aβ levels that subsequently accelerates the onset of related behavioural and histopathological alterations, and aggravates them. The early onset of these deficits makes $5XFAD_{hom}$ a well-suited model for preclinical studies within a short time frame. Moreover, due to the strongly increased levels of Aβ, the model might especially facilitate the analysis of intracellular Aβ pathology at very early time points.

5. SUMMARY

The present study aimed at further investigation of two transgenic mouse models of Alzheimer's disease (AD): Tg4-42 and 5XFAD.

The transgenic mouse model Tg4-42 was recently developed in our group and displays the variety of typical AD features. It does not possess any mutations but overexpresses N-terminally truncated $A\beta_{4\text{-}42}$. Thereby, it is likely that Tg4-42 represents the model for sporadic form of AD. The Tg4-42 model is unique because of its massive neuron loss and severe cognitive deficits induced through intraneuronal accumulation of N-terminally truncated $A\beta_{4\text{-}42}$ without amyloid plaque formation.

So long as current drug treatments have only modest effects on the symptomatic course of AD, environmental interventions are of great interest in order to prevent AD or delay its onset. In ***the first project*** of my study it was shown that environmental enrichment, combining physical activity and cognitive enhancement, leads to an amelioration of neuronal loss in Tg4-42. Furthermore, the evidences were provided that physical activity triggers neurogenesis in Tg4-42 and rescues it to the wild-type levels.

For this study, the group of Tg4-42_{hom} mice was exposed to the enriched environment (EE) starting at age of 2 months. At the age of 5 months, mice were tested for hippocampus-dependent memory, motor abilities, and anxiety levels (projects of my colleagues). Their cerebral tissue was subsequently analysed for neurogenesis and neuronal loss in the dentate gyrus (DG) (present study). Therefore, group of Tg4-42_{hom} mice housed under EE conditions was compared to the age-matched Tg4-42_{hom} group and wild-type group from the standard housing (SH). Unbiased design-based stereology revealed that EE ameliorates neuron loss Tg4-42_{hom} mice. The number of DG cells in 6-month-old Tg4-42_{hom} mice was restored to the levels of the wild-type mice. In order to quantify the neurogenesis, immunohistochemistry with doublecortin (DCX) has been performed. DCX is a microtubule-associated protein expressed in all migrating neuronal precursors in central nervous system. Using stereology, DCX-positive new-born neurons were counted in the DG. The analysis of the standard housed Tg4-42_{hom} mice revealed a very low amount of DCX-positive cells with impaired dendritic arborization in comparison with SH wild-types. Quite the contrary to SH-housed Tg4-42_{hom} mice, the EE-housed mice showed strongly increased number of DCX-positive cells with much more pronounced processes. EE was able to restore the number of new-born neurons in Tg4-42_{hom} mice almost to the levels of SH wild-types.

The results of this study should be considered together with the results from other colleagues from our research group providing evidences that enhanced physical activity improves motor abilities and counteracts declining memory performance.

In summary, the results obtained here might indicate the importance of environmental enrichment, in particular increased physical activity, as a potential strategy in the prevention of dementia. They also validate Tg4-42 mouse model as a well-suitable model for investigation of the sporadic form of AD.

The focus of ***the second project*** laid on the 5XFAD mouse model. The 5XFAD is a widely used model, which is based on the expression of mutant amyloid precursor protein and presenilin-1 genes, and represents thereby a mouse model for familial AD. It is a typical model for early plaque formation, intraneuronal Aβ aggregation, neuron loss and robust behavioural deficits.

This project aimed at investigation the gene dosage dependence of the neuropathological features in 5XFAD. Therefore a homozygous 5XFAD line was created to elevate transgene expression and Aβ production. In order to evaluate the effect of increased gene dosage on the amyloid pathology and axonopathy, hemizygous and homozygous 5XFAD mice were compared. Sagittal brain sections of hemizygous and homozygous 5XFAD mice at 2, 5 and 9 months of age were stained with an Aβ[N] antibody, which detects an N-terminal Aβ epitope, and the amyloid plaque load was quantified in the cortex, hippocampus (hippocampal region CA and DG), retrohippocampal region (subiculum)) and thalamus. Furthermore, dystrophic neurites were counted in the pons and spinal cord in order to evaluate the extent of axonopathy. In the present study, it was shown that the levels of extracellular amyloid formations are significantly elevated in the $5XFAD_{hom}$ groups compared to the respective $5XFAD_{hem}$ groups in all analysed brain regions at all time points. 3- to 7-fold increases Aβ plaque load were found at the 2- and 5-month-stages. 9-month-old mice showed a lesser increase in Aβ plaque load that might reflect a saturation effect. Along with the aggravation of the amyloidosis, it was demonstrated that 9-month-old $5XFAD_{hom}$ mice show severe axonal degeneration in the pons and the spinal cord compared to age-matched heterozygous mice.

The results of this study should be considered together with the results from my colleagues, especially from Bernhard Richard, who performed a phenotypical characterisation of $5XFAD_{hom}$ mice and investigated a behavioural phenotype with deficits in reference learning and memory: besides the described acceleration of amyloid plaque pathology and strongly increased axonal degeneration, it was shown that the development of memory deficits and behavioural impairment was also accelerated.

Thereby, homozygous 5XFAD mice represent a model with several advantages comparing with the heterozygous mice. Namely, $5XFAD_{hom}$ mice develop amyloid pathology much more rapidly as well as a neurological phenotype. Bred to homozygosity 5XFAD mice show a strongly reduced age of onset of behavioural symptoms and neuropathological alterations. These advantages can speed up preclinical studies. In conclusion, the results obtained in this study have broadened the knowledge about the 5XFAD mouse model and further validated this model as an appropriate model for investigation of AD and for analysis of Aβ species, memory and behaviour.

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