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Effect of CNTF Derived Peptide, P021 on Cognition and Pathology in 3xTG-AD Mouse Model of Alzheimer's Disease

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EFFECT OF CNTF DERIVED PEPTIDE, P021 ON COGNITION AND PATHOLOGY IN 3xTG-AD MOUSE MODEL OF ALZHEIMER’S DISEASE

By
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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Effect of CNTF derived peptide, P021 on cognition and pathology in 3xTg-AD mouse model of Alzheimer’s disease

By

Narjes Baazaoui

Advisor: Dr Khalid Iqbal

Studies described in this thesis deal with the preventive effects of a neurogenic/neurotropic peptidergic compound, P021, on neurogenesis and synaptic deficits, neurodegeneration, cognitive impairment, and Aβ and tau pathologies in a 3xTg-AD mouse model of Alzheimer’s disease (AD).

Background: AD is a chronic progressive neurodegenerative disease. Its multifactorial nature and the heterogeneity make its treatment especially challenging. Although it is a major burden in society, at present there is no drug that can stop or slow down the progression of the disease. Currently, the only available treatments are symptomatic and for mild to severe stages. The development of a drug that can prevent AD at its early stages would be of major importance. The use of neurotrophic factors mimetic for the treatment of AD is an exciting therapeutic strategy. It focuses mainly on boosting synaptic plasticity and neurogenesis which lead to shifting the balance from neurodegeneration to regeneration of the brain as well as preventing Aβ and tau pathologies. Herein, I present the preventive effects of P021 treatment in 3xTg-AD mice that is initiated very early in the disease during the period of synaptic compensation and is continued for the lifespan of the animal.
Aims: The specific aims were: 1) to study the presence of the synaptic compensation phenomenon in the brain as a self-repairing mechanism in 3xTg-AD mice; 2) to study the preventive effects of P021 on cognitive deterioration when administered during the compensation period; 3) to study the preventive effects of P021 on amyloid beta (Aβ) and tau pathologies; and 4) to study the effect of P021 on synaptic deficit, neuronal degeneration and neurogenesis.

Methods: A battery of behavioral tests was conducted to assess the cognitive performance in 3xTg-AD mice with and without treatment with P021 at different disease stages. Immunohistochemical and biochemical analyses were performed to determine the levels of synaptic protein expression as well as Aβ and tau pathologies at different time points that correspond to different stages of disease progression. Neurodegeneration was studied immunohistochemically with Fluorojade C staining. Neurogenesis was studied immunohistochemically with DCX (double cortin) and Ki-67 staining.

Results: The 3xTg-AD mice at the age of 12 weeks were found to be cognitively impaired and showed a decrease in multiple synaptic and neuronal markers. This decrease was compensated by the brain until ~16 weeks of age. Beyond 16 weeks the brain was found to fail to compensate for the synaptic deficit. P021 intervention, started at 14 weeks of age, prevented cognitive impairment 9 months post-treatment, as tested by the Morris Water maze task. At 15-17 months post-treatment P021 was able to rescue short-term spatial reference memory as well as episodic memory, as determined by the novel object location and the novel object recognition tasks. The treatment with P021 also prevented Aβ and tau pathologies during 9-18 months post-treatment. P021 was able to rescue synaptic deficit and neurodegeneration 9-18 months post-treatment and boost neurogenesis at 9 months post-treatment. P021 treatment increased survival from 41% in
3xTg-AD-vh to 87% in 3xTg-AD-P021 mice. In the entire study I did not find any severe side effects of P021 including loss of appetite or body weight.

Conclusions: Early intervention with P021 during the period of synaptic compensation of the brain was successful in preventing cognitive impairment in 3xTg-AD mice. The P021 treatment prevented synaptic and neurogenesis deficits, neurodegeneration, and Aβ and tau pathologies. These findings provide a proof of principle of the potential therapeutic effect of P021 on several major features of AD.

Key Words: Aβ pathology, Alzheimer’s disease, brain derived neurotrophic factor, cognitive impairment, neurodegeneration, neurogenesis, neurotrophic factor, mortality, synaptic plasticity, tau pathology, therapeutic prevention, 3xTg-AD.
Dedication

This work is whole heartedly dedicated,

To my dear father Brahim for his strong emotional and moral support. He is the one who pushed me during the whole period of my doctoral studies to continue my work despite many difficulties that I was going through when he said “his only wish before he dies is to see me graduate and become a doctor, (Ph.D.)”.

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To my beloved husband Riadh and my son Mohammed Moetaz and I wanted to tell them that I am so sorry that I had to be separated from them for one year and a half and that their sacrifice is

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getting paid off. I especially want to thank my husband for taking good care of my son while I am away and for his continuous support for my career. May God keep us together till the last day of our lives.

To all my friends

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After spending five years doing my doctoral studies at IBR working day and night, I would say that science is fun. Although all the hard moments of failed experiments and the stress to get work done, the stress to spend the whole night in the lab I would say that I really enjoyed it. Being a doctoral student every day you discover that you have some strength that you never knew about yourself. I discovered that I could write my whole dissertation within 23 days, and that I am strong enough to do it!

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List of Abbreviation Used

AD-P tau; hyperphosphorylated tau
AD, Alzheimer’s disease
API, Alzheimer’s prevention initiative
Aβ, amyloid beta
APP, amyloid precursor protein
ALS, amyotrophic lateral sclerosis
APOE4, Apolipoprotein E4
BBB, blood brain barrier
BDNF, brain derived neurotrophic factor
CNS, central nervous system
CNTF, ciliary neurotrophic factor
CA1, cornu ammonis 1
CA3, cornu ammonis 3
DG, dentate gyrus
DIAN, Dominantly Inherited Alzheimer’s Network
DCX, double cortin
FAD, familial Alzheimer’s disease
FGF-2, fibroblast growth factor 2
FDA, Food and Drug Administration
fMRI, functional magnetic resonance imaging
GDNF, glial derived neurotrophic factor
GSK3β, Glycogen synthase kinase 3 beta
HD, Huntington’s disease
IGF-1, insulin growth factor-1
IGF-R1, insulin growth factor receptor 1
IR, insulin receptor
ICV, intracerebroventricular injection
JAK, Janus kinase pathway
LIF, leukemia inhibitory factor
mRNA, messenger RNA
MAP, microtubule associated proteins
MCI, mild cognitive impairment
NGF, nerve growth factor
NPS, neural progenitor cells
NFT, neurofibrillary tangles
PD, Parkinson’s disease
PNS, peripheral nervous system
PS1, Presenilin 1
PS2, Presenilin 2
PT, probe trial
STAT-3, Signal transducer and activator of transcription 3
SGZ, subgranular zone
SVZ, subventricular zone
TBI, traumatic brain injury
3xTg-AD mouse, triple transgenic mouse model of AD
3xTg-AD-vh, 3xTg mice treated with vehicle diet
3xTg-AD-P021, 3xTg-AD mice treated with P021 diet
WT, wild type
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Introduction

Alzheimer’s disease (AD) is the sixth leading cause of death in the United States and the most common form of dementia; it accounts for 60-80% of dementia cases. Although AD is known as a disease of old age, with more than 95% of AD sporadic cases occurring at an age of >65 years, the remaining <5% are familial cases and have early onset, usually around the age of 40 to 50 years. AD is a progressive neurodegenerative disease with a slow memory loss at early stages followed by severe cognitive impairment and memory loss at later stages along with personality changes and susceptibility to infectious diseases such as pneumonia, which is the major cause of death at later stages. It is a non-linear disease in which the first brain changes occur more than a decade before the appearance of clinical symptoms. AD is characterized by two histopathological hallmarks, β-amyloidosis and neurofibrillary degeneration, which are the biological basis for dementia. The oligomeric forms of tau and amyloid beta (Aβ) are believed to be most toxic to neurons because they, respectively, sequester normal tau and bind to cell surface receptors and contain β conformation that ends up in β sheets which make them resistant to degradation. Aβ accumulation is believed to disrupt the communication between neurons through the synapses, which leads to synaptic death and later to neuronal death. The intracellular accumulation of tau as neurofibrillary tangles (NFT) is believed to disrupt the axonal transport, which leads to synaptic death followed by neuronal death. Indeed, the brain atrophy seen in AD is consistent with neurodegeneration seen in patients with severe AD.

Because of the increase in lifespan in the developed countries, AD has increasingly become a major health problem and burden to society. Worldwide, AD currently affects 44 million people and this number is expected to increase to 135 million by 2050 with the aging of the baby boomer generation. The costs for health care in the US alone are estimated to be about
$217 billion (Alzheimer's Association 2015) in addition to social and emotional costs. Because AD has a multifactorial nature and the neuropathological insults that, at present, cannot be reliably detected in living individuals start more than a decade before the appearance of the first clinical symptoms, it makes it difficult to develop a drug that can stop or at least slow down the progression of the disease. Currently there is no treatment for AD. The six approved drugs are able only to temporarily improve the clinical symptoms; they do not stop or slow down the disease. The majority of the approved drugs for AD are cholinesterase inhibitors such as Tacrine (rarely prescribed today), Donepezil for all AD stages, Galantamine for mild to moderate stages, and Rivastagmine for mild to moderate stages. Memantine is an NMDA receptor antagonist and has beneficial symptomatic effect only during moderate to severe stages of AD. Namzaric is a combination of donepezil and memantine. All of these drugs act by increasing neurotransmitter release. Tacrine, Galantamine and memantine have also been reported to increase neurogenesis (Jin et al., 2006). From 2002-2012, the only new drug approved by the Food and Drug Administration (FDA) was memantine. Namzaric was approved in late 2014. A major problem that faces drug development is that it is a lengthy process with a 95% chance of failure, and the average time for a development of a drug from the discovery to approval in the market requires an average of 10-15 years (Schneider et al., 2014).

Because of the repeated failures in the development of a drug to treat AD, several therapeutic approaches have been developed in recent years in an attempt to design an effective drug for AD. There are two major approaches; the first one is to inhibit neurodegeneration and disease progression and the second one is to reverse neurodegeneration and prevent cognitive impairment by boosting the regenerative capacities of the brain. In most disease modifying strategies the first approach used to attempt to inhibit neurodegeneration was to remove toxic
protein aggregates such as Aβ aggregates, Aβ plaques, tau aggregates and NFT. However, the phase III clinical trials of the drugs targeting Aβ showed that this approach is ineffective at least in patients with an advanced stage of the disease. This has led to conducting human clinical trials with Aβ passive immunotherapy at mild and prodromal stages of the disease and to tau-based therapeutic approaches, especially immunotherapies (Iqbal et al., 2016). The other promising approach in developing and designing AD drugs is to reverse synaptic loss and neurodegeneration and to boost neurogenesis, focusing mainly on treating the pathophysiological features of the disease rather than directly targeting Aβ and tau pathologies. Indeed, previous studies showed that synaptic loss is more tightly related to neurodegeneration and the progression of the disease than NFT or Aβ plaques (Terry et al., 1991). The use of a small neurotrophic peptide mimetic that has neurogenic/neurotrophic effect, is able to cross the blood-brain barrier (BBB), is bioavailable and biostable, and is safe, with minimal or no side effects, represents a promising strategy to reverse neurodegeneration. This strategy has a wide therapeutic window compared to toxin clearance approaches and, because of the neurogenic/neurotrophic effect, can be used even at a later stage of the disease to stop or slow down its progression. Because of the lack of early diagnosis and the unavailability of efficient biomarkers, AD is discovered only after significant pathological changes have happened in the brain. Indeed, in individuals with disease-causing autosomal dominant mutations, who are at a very high risk to develop AD, Aβ deposition starts 15-20 years before the appearance of any clinical symptoms (Lu et al., 2013; Sperling et al., 2014).

In our lab we adopted the approach of boosting the regenerative capacity of the brain using a neurogenic/neurotrophic peptidergic compound mimetic, named P021 (Ac-DGGLG-NH2). P021 is derived from the most active region of the ciliary neurotrophic factor (CNTF) (Li
et al., 2010). It has a plasma half life of > 3 h, is 100% stable in the intestinal fluid during 2 h, and ~90% stable in gastric juice during 30 min (Kazim et al., 2014). P021 is adamantylated on its C-terminal to protect it from degradation by carboxypeptidases and increase its blood brain barrier (BBB) permeability. Previously we showed that P021 increases neurogenesis and neuronal plasticity (Blanchard et al., 2010b, Bolognin et al., 2012, Kazim et al., 2014). The neurogenic and neurotrophic effects of P021 are due to its competitive inhibition with Leukemia inhibitory factor (LIF) through the decrease in the phosphorylation of pSTAT3 (Li et al., 2010, Kazim et al., 2014). It decreases tau phosphorylation through the increase in BDNF expression mainly through enhancing its transcription (Bolognin et al., 2012, Bolognin et al., 2014). The increase in BDNF expression leads to an increase in the phosphorylation of GSK3β at serine 9 which leads to its inactivation. Since GSK 3β is the major serine/threonine kinase that phosphorylates tau at many different sites such as Ser199, Ser202, Thr205, Ser396, and Ser404, its inactivation results in a decrease in the level of phosphorylation, hence to a global decrease in tau pathology. Besides, P021 also has an effect on decreasing Aβ pathology through the decrease in the generation of Aβ (Kazim et al., 2014). This P021 effect is thought to occur also through the inhibition of GSK3β, which is also implicated in Aβ mediated neuritic damage (DaRocha-Souto et al., 2012), APP processing and Aβ deposition (Phiel et al., 2003).

Several reports in the literature emphasize the presence of a compensatory mechanism during the early stages of AD to slow down neurodegeneration, synaptic loss and cognitive deterioration. The compensatory phenomenon was shown to work by enhancing neurogenesis to replace the lost neurons (Li et al, 2008) and increase the production of neurotrophic factors such as the brain derived neurotrophic factor (BDNF) (Faria, et., 2014) or synaptic plasticity either by an increase in the level of expression of synaptic proteins or in the size of the surviving synapses
to compensate for synaptic loss (Scheff et al., 2006). It is believed that this compensation is present in healthy aging, but it is defective in AD and it occurs only during early stages where there is minimal brain insult (Bertoni-Freddari et al., 1988, Barnes, 1994, Brown et al., 1998, Mukaetova-Ladinska et al., 2000). Probably that is why there is a slow progression of the disease. In contrast, once the brain damage reaches a certain threshold, the compensation phenomenon fails and the cognitive deterioration and clinical symptoms show a drastic and dramatic decline. Therapeutic intervention during the compensation period is believed to boost compensation and help prevent cognitive impairment, neurodegeneration and synaptic loss.

The specific objective of this study was (i) to investigate the presence of the compensation phenomenon at a very early stage of the disease akin to AD in 3xTg-AD mice and (ii) to start the treatment with P021 when the compensatory phenomenon is still effective to investigate the effect of the compound on the prevention of the disease. The specific aims were: 1) to study the presence of the synaptic compensation phenomenon in the brain as a self-repairing mechanism in 3xTg-AD mice; 2) to study the preventive effects of P021 when administered during the compensation period on cognitive deterioration; 3) to study the preventive effects of P021 on amyloid beta (Aβ) and tau pathologies; and 4) to study the effect of P021 on synaptic deficit, neuronal degeneration and neurogenesis.

I present and discuss the data of this study at four different time points: the first one is at 3 months of age before the treatment with P021; the other time points are 9 months (12 months of age), 15 months (18 months of age) and at 18 months after treatment (21 months of age).

This work is presented as a chapter-based dissertation: Introduction, Background (Chapter 1), Material and Methods (Chapter 2), synaptic compensation study in 3xTg-AD mice (Chapter 3), the effect of early intervention with P021 on cognitive impairment (Chapter 4), on
Aβ and tau pathologies (Chapter 5), and on neuroregeneration and neurodegeneration (Chapter 6), and a general discussion (Chapter 7).

With this research I am aiming to answer the following questions: 1) would early treatment with P021 be effective on boosting the compensatory mechanism? 2) Would early intervention be effective in preventing cognitive impairment? 3) Would it prevent Aβ and tau pathologies? And 4) would it prevent neuronal and synaptic loss? Finally, 5) does P021 have the potential to be considered for further drug development as a promising novel therapeutic approach for AD?
Chapter 1. Background

1.1 Alzheimer’s disease classification, prevalence, and public cost

1.1.1 AD classification

AD is classified into two major categories: 1) The sporadic or late onset form, which represents the vast majority of AD cases (more than 95%) and tends to develop at the age of > 65 years with an unknown etiology; and 2) the familial or early onset form, which represents the remaining ~5% of cases. The familial AD is genetically inherited through mutations in one of the three genes, the amyloid precursor protein (APP), presenilin 1 or presenilin 2. Generally early onset AD or the inherited form tends to progress faster and follows a Mendelian pattern of inheritance (Reitz and Mayeux, 2014). Mutations in APP or presenilin 1 are associated with complete penetrance, meaning that any individual who has one of the mutations is guaranteed to have an early onset of the disease. Presenilin 2 mutation has only 95% penetrance, thus not everyone who has the mutation will develop AD. Individuals that have mutation in any of these genes tend to have an early onset of AD, sometimes as early as 30 years of age (Alzheimer's Association 2015).

1.1.2 Prevalence and epidemiology

AD is the major cause of dementia; it represents 60-80% of all dementia cases with about half involving only AD. The incidence of AD increases with age, doubling every 5 years. The prevalence also increases with age from 3% at the age of 65 to ~50% at the age of 85 or older. Because of the decrease in birth rate and the increase in life expectancy, the percentage of elderly people in the US is expected to increase from 12.4% (35 million) in 2000 to 19.6% (71 million) in 2030. Worldwide the number of people that are 65 years or older is expected to increase more than double from 420 million in 2000 to 973 million in 2030 (Castellani et al.,
In the US alone there are around 5.3 million people that have AD, with 5.1 million aged 65 years or older and approximately 200,000 with early onset. This number is projected to increase up to ~15 million in 2050. Women represent almost two thirds of the AD cases in the US. Indeed, out of the 5.1 million affected, 3.2 million are women. Currently in the US every 67 seconds there is a new case of AD and by 2050 it will be every 33 seconds. With the increase in life expectancy and the aging of the baby boomer generation, the number of elderly people may even increase. Because aging is a high risk factor for AD, the number of people who develop AD would even increase.

AD is the sixth leading cause of death in the US and the fifth leading cause of death in individuals 65 years and older. Between 2000-2013 deaths ascribed to AD increased by 71% while deaths attributed to heart disease (the most frequent cause of death) decreased by 14%. In the US AD rose from the 25th most burdensome disease in 1990 to the 12th most burdensome disease in 2010 (Alzheimer's Association 2015). In 2005, the highest prevalence of dementia was recorded in the US and Western Europe, affecting 6.4 and 5.4% of the population at age 60, followed by Latin America and China (Mayeux and Stern, 2012).

1.1.3 Public cost

The financial cost of AD is large. Indeed, the lifetime cost per AD person is estimated to be $174,000 (Castellani et al., 2010). In 2015, the estimated total health care and hospital services for people that are 65 years or older with dementia was $226 billion. The contribution from family members and other unpaid caregivers was estimated to be 17.9 billion hours of caregiving, which is valued at more than $217 billion. By 2050 the national cost of AD could reach $1 trillion. Beyond financial costs AD also causes major social and emotional distress to the patient and his/her family (Alzheimer's Association 2015).
1.2 AD diagnostics and symptoms, risk factors and preventive factors

1.2.1 Diagnostics and symptoms

*Diagnostic criteria*

Diagnostic criteria for AD were proposed in 1984 by a group of experts put together by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association. These criteria combine both clinical and neuropathological patterns to classify AD as possible (for typical clinical syndrome without intervening issues), probable (for diagnosis complicated by other disorders intervening with dementia) and definite (for autopsy confirmed disease) AD and they were considered reliable for diagnosing AD for more than three decades. AD is considered as a continuum with a broad spectrum starting with an asymptomatic stage at early adulthood to definite AD late in life at the age of 65 years or older. The pathological features of AD include not only Aβ and tau pathologies but also synaptic deficit, neuronal loss, unsuccessful neurogenesis and microgliosis (Mayeux and Stern, 2012, Reitz and Mayeux, 2014).

*Symptoms*

The early symptoms of AD are mainly difficulty remembering new conversations, names or events. It is often accompanied by depression and apathy. This mild cognitive impairment (MCI) is an intermediate stage between the expected cognitive decline of normal aging and the more serious decline of dementia. It can involve problems with memory, language, thinking and judgment that are greater than normal age-related changes but does not interfere notably with the activities of daily life (Gauthier et al., 2006). In AD this MCI stage leads to symptoms that are mainly related to disorientation, difficulty swallowing, walking or carrying a conversation, poor judgment and personality change (Fig. 1). How fast the symptoms change from mild to moderate to severe varies among patients (Alzheimer's Association 2015).
Fig. 1: Model of the clinical trajectory of AD (Sperling et al., 2011).

1.2.2 Risk factors

Age

Age is a major risk factor to develop AD. Usually people who are 65 years or older are more susceptible to develop AD. However, age alone does not cause AD and AD is not part of normal aging (Alzheimer’s Association 2015).

APOE4

Inheritance of the APOE4 allele is a major genetic risk factor for developing AD. The APOEε4 allele is located on the chromosome 19q13. APOE is a lipid-binding protein that is expressed in three isoforms in humans: APOE2, APOE3, and APOE4. These isoforms are coded by the same gene locus and consist of 299 amino acids; the only structural difference is a single amino acid change. APOE3 has Cys-112 and Arg-158, whereas APOE4 has arginines at both sites, and APOE2 has cysteines at both sites. APOEε2 has a frequency of ~8.4% in the general
population and it is believed to have a protective effect against AD. APOEε3 is the most common allele (frequency of ~77.9%) and it is believed to have a neutral effect in developing AD. APOEε4 is present in ~14% of the population and in ~40% of the late onset of AD cases (Wikipedia). People who inherit one copy of the allele APOEε4 have a three fold higher risk of AD while people who have two copies of the APOEε4 have 8-15 fold higher risk of having AD and they are more likely to have it than people who carry the APOEε2 or the APOEε3 alleles. Approximately 2% of the US population carry APOEε4. Out of all the cases of AD 40-60% of people who are diagnosed with AD carry one or two copies of APOEε4 (Alzheimer's Association 2015). Each copy of the APOEε4 allele lowers the age of onset of AD by 6-7 years. However, the presence of the ε4 allele is not sufficient or necessary to develop AD (Reitz and Mayeux, 2014).

Other factors

An increasing number of studies link heart diseases to brain diseases, which is why cerebrovascular diseases such as hemorrhagic infarcts, small and large ischemic infarcts and vasculopathies are considered to be a risk for having AD. Stroke increases the risk of having dementia by 4-12 times and the mechanism is unknown. High blood pressure in mid-life has been shown to increase the risk of cognitive impairment and dementia in later life. Having type II diabetes doubles the risk of having AD for as yet unknown reasons. The increase in cholesterol level has been controversial as being a risk factor for AD. However, the increase of cholesterol in mid-life is suspected to have a damaging effect in later life (Reitz and Mayeux, 2014). The risk of AD is 2.3 times higher in people who experience moderate traumatic brain injury (TBI) and 4.7 times higher for people who experience severe TBI compared to non injured individuals (www.alz.org). People with mild cognitive impairment (MCI) are at higher risk for developing
AD, especially if the MCI is accompanied by memory problems, which will more likely lead to early AD. 10-20% of people that are more than 65 years old have MCI and among those who had serious symptoms 15% develop early AD. However, some MCI cases can reverse to normal cognition or the MCI can remain stable and not proceed to AD (Alzheimer's Association 2015).

1.2.3 Preventive factors:

Exercise

It has been well documented that physical exercise increases the level of the brain derived neurotrophic factor (BDNF) and increases neurogenesis (van Praag et al., 1999) and neuronal plasticity. Exercise may also increase brain vascularization (Black et al, 1990), neuronal survival and resistance to brain insult (Carro et al., 2001).

Diet

Recently it was shown that the Mediterranean diet is associated with a lower risk of developing AD and a lower mortality rate in AD patients. The Mediterranean diet is mostly composed of vegetables, cereals, fish, a low to moderate intake of dairy products and a low to moderate intake of poultry and meat (Castellani et al., 2010). In contrast, the consumption of a high fat diet was associated with an increase of cholesterol and an increased risk of vascular disease in the brain (Mayeux and Stern, 2012).

Brain and cognitive reserve

The fact that some patients have AD neuropathological lesions that are not accompanied by cognitive impairment led to the suggestion that these people are resilient and they have high cognitive or brain reserve. Brain reserve encompasses both brain size and cognitive reserve. The cognitive reserve refers to the ability of the brain to use alternative neuronal networks or cognitive strategies to overcome the cognitive impairment and it was associated with a higher
level of education (Alzheimer’s Association 2015). Thus, a high brain reserve, it is suggested, could exert a compensatory and a protective effect against AD. Compensation would be effective for a long period of time to protect from cognitive decline. However, once a certain threshold of damage was reached, the compensation would fail and the cognitive decline would be more rapid and drastic compared to AD non-resilient patients (Sperling et al., 2014). In a large longitudinal cohort study consisting of AD, amnestic mild cognitive impairment patients (aMCI) and healthy controls, a larger intracranial volume was found to attenuate the effect of atrophy and APOE4 in aMCI but it did not correlate with the severity of clinical symptoms or worsening of atrophy. However, this protective effect was found to be efficient only during early stages of AD. Once a neurodegeneration threshold is reached, brain size is no longer protective (Guo et al., 2013). This argues in favor of a role of brain reserve in compensation for neuronal loss instead of neuroprotection.

1.3 The three common hypotheses of AD

The exact cause of AD remains to be established. Several hypotheses concerning the etiopathogenesis have evolved over the years but to date none of them have been found to explain all aspects of the disease. The three major hypotheses are the cholinergic, Aβ cascade, and tau hypotheses, as follows.

1.3.1 Cholinergic Hypothesis

Previously AD was considered to be a cholinergic disease associated with severe loss of central cholinergic neurons and the level of cognitive impairment was correlated with the level of cholinergic loss. Indeed, the cholinergic hypothesis states that the cognitive impairment in AD is caused by the degeneration of basal forebrain cholinergic neurons due to aberrant signaling of the neurotrophin P75 receptor through the increase in proNGF level and decrease in NGF level.
in AD patients. The increase in proNGF level was shown to stimulate cell death through the P75 receptor (Podlesniy et al., 2006). This hypothesis led to the development of cholinomimetics like Tacrine, Donepezil and Rivastigmine that are being used for the symptomatic treatment of AD.

1.3.2 Aβ hypothesis

APP is an integral membrane protein that acts as a signaling receptor. It is produced due to alternative splicing of its transcripts as three different isoforms: APP\textsubscript{695}, APP\textsubscript{751}, and APP\textsubscript{770}. The three mRNA isoforms are present in almost every tissue in different ratios. It is the mRNA and the protein expression of APP\textsubscript{695} that is more prominent in neurons. APP is metabolized through two different pathways: amyloidogenic and non amyloidogenic (Fig. 2). In the non amyloidogenic pathway APP is first cleaved by α-secretase in the Aβ region between Lys16 and Leu17 and the resulting carboxy-terminal fragment is further cleaved by γ-secretase enzyme. This pathway precludes the production of Aβ. However, in the amyloidogenic pathway APP is sequentially cleaved by β- and γ-secretases to produce a soluble monomeric Aβ fragment. The most commonly produced Aβ isoforms are Aβ1-40 and Aβ1-42. Aβ1-40 is more prone to deposit in brain blood vessels, as seen in cerebral amyloid angiopathy, whereas Aβ1-42 is more prone to form insoluble aggregates because of its hydrophobic nature. Since Aβ is a normal metabolite of APP it is believed that an imbalance between the rate of production and clearance of Aβ is what leads to Aβ plaque deposition. The enzyme BACE1 is responsible for β-secretase activity, while PS1 and PS2 are part of the γ-secretase complex. The α-secretase activity is mediated by the ADAM family, especially ADAM10 and ADAM17 (Hicks et al., 2012, Tarasoff-Conway et al., 2015).

Certain APP mutations that are causative of familial Alzheimer’s disease (FAD) also result in the increase of Aβ synthesis. Since PS1 and PS2 have γ-secretase activity, certain
mutations in either of these proteins can also increase Aβ production. However, it is well established that Aβ load does not correlate with cognitive impairment in AD. The Aβ hypothesis then places the accumulation of Aβ as the major cause and the earliest key event in the AD pathophysiological process, followed later on by NFT and neuronal loss. However, this hypothesis remains controversial and challenged mainly because all the clinical trials based on anti-Aβ therapies failed to treat AD (Sperling et al., 2014).

1.3.3 Tau hypothesis

The tau hypothesis is that abnormally hyperphosphorylated tau is causative of the disease. In AD the dementia correlates with the density of tau pathology. For instance, the severity of dementia correlates better with the increase in the accumulation of NFT and the level of hyperphosphorylated tau in the CSF correlates better with cognitive impairment (Rafii and Aisen, 2015). It is hypothesized that due to aberrant signaling of tau protein kinases and/or phosphatases, tau becomes hyperphosphorylated and aggregated into NFT, leading to abnormal cellular trafficking and disruption of the microtubule-based cytoskeleton, followed by cell death. After cell death the oligomeric forms of tau are released to the extracellular environment, which stimulates microglial cells, leading to further prion-like spread of tau pathology and neurodegeneration. So far the closest approximation to cognitive impairment in AD is the tau hypothesis. The strongest support for the tau hypothesis comes from the fact that certain mutations in the tau gene cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17). Furthermore, several tauopathies are characterized by accumulation of abnormally hyperphosphorylated tau in the absence of Aβ pathology. NFT and neuropil threads are common pathological features shared between various tauopathies such as frontotemporal dementia, Pick’s disease, progressive supranuclear palsy and corticobasal
degeneration (Brunden et al., 2009). The fact that all tauopathies share abnormal hyperphosphorylated tau inclusions as a common pathological feature that is followed by neurodegeneration and clinical phenotype strongly supports the idea that tau by itself can cause neurodegeneration without the presence of Aβ.

1.4 Underlying pathologies
The neuropathological hallmarks of AD are grouped into so-called “negative” and “positive lesions”. The negative lesions mainly represent synaptic and neuronal loss and the positive lesions mainly represent Aβ plaques and NFT. Plaques are primarily localized in the cerebral cortex and NFT are primarily localized in the limbic and cortical association areas (Serrano-Pozo et al., 2011a).

1.4.1 Aβ plaques
Amyloid beta (Aβ) plaques (Fig. 2) are extracellular, spherical deposits of Aβ (10-200 μm in diameter) in the cerebral cortex. They are classified as dense core or diffuse plaques. Dense-core plaques are thioflavin S and congo-red positive, usually associated with dystrophic neurites, reactive astrocytes and synaptic loss, and they are considered to be pathological. They are mainly composed of Aβ 42 and their presence is generally associated with cognitive impairment. Diffuse plaques are thioflavin S and congo-red negative, with amorphous shape, not accompanied with dystrophic neurites or synaptic loss, and they are not considered for a pathological diagnosis of AD because they are also found in the brains of healthy individuals. The spatio-temporal pattern of the staging of the plaque pathology, unlike NFT, is far less predictable but it is generally classified into three stages: first in isocortical areas, then in allocortical or limbic areas, and finally in subcortical areas (Serrano-Pozo et al., 2011a). Aβ
deposition does not correlate with the severity of cognitive symptoms. In fact, it was shown previously that the Aβ deposition reaches a plateau in the isocortex right after the onset of cognitive symptoms even in the preclinical stages of the disease (Ingelsson et al., 2004, Serrano-Pozo et al., 2011b).

Fig. 2. App processing pathways: amyloidogenic and non-amyloidogenic pathways (Hicks et al., 2012).

1.4.2 NFT

Tau is a microtubule associated protein that is abundant in neurons; its main function is to bind to and stabilize microtubules to facilitate axonal transport. In the normal physiological condition tau is seen mainly localized in the axons and it is phosphorylated by several kinases to destabilize microtubules and promote plasticity. Normal adult human brain tau contains 2-3 moles of phosphate, while in the AD brain tau is 3-4 times more hyperphosphorylated (Iqbal et al., 2010). Tau hyperphosphorylation happens either because of hyperactive kinases or
hypoactive phosphatases. Pathological tau in AD patients is found to be localized in the somatodendritic compartment. Hyperphosphorylation of tau leads to destabilization of microtubules and sequestration of normal tau by the pathological protein and its self-assembly into paired helical filaments (PHF) as neuropil threads and NFT. NFT (Fig. 3) are intraneuronal aggregates of hyperphosphorylated tau and misfolded tau and they are seen after neuronal death as “ghost” tangles in the extracellular space. NFT have a spatiotemporal spread that correlates with the severity of cognitive impairment and they parallel synaptic and neuronal loss. The topographic staging of AD is used for the diagnosis of AD. Early NFT formation starts in the entorhinal/perirhinal cortex, followed by the hippocampus, the association cortex and finally the primary cortex (Braak and Braak, 1991, Serrano-Pozo et al., 2011a, Glass and Arnold, 2012). NFT are classified as pre-NFT with diffuse and sometimes punctuate staining in normal-looking neurons. Mature or intraneuronal NFT are found in the cytoplasm and they push the location of the nucleus to the periphery. The shape of dendrites and the proximal axon looks distorted. Extracellular or ghost NFT are found when tangle-bearing neurons are dead and they are identified by the absence of a nucleus and stainable cytoplasm (Serrano-Pozo et al., 2011a). NFT are localized mainly in the neuron clusters of feed-forward and feed-back connections between cortical and subcortical areas, leading to a disconnection between the hippocampus and the neocortex and between the neocortical association areas. This disconnection results in the impairment of intellectual functioning (Armstrong, 1993, Terry et al., 1994).
Fig. 3: Aβ plaques and NFT in a Bielchowsky silver stained section of AD hippocampus. From Iqbal et al., in “An Atlas of Alzheimer’s Disease”; edited by Mony J. DeLeon and Heiko Braak, Parthenon Publishers Group, June 15, 1999.

1.4.3 Changes in synaptic plasticity

Synaptic loss

The process of neurodegeneration is mainly accompanied by synaptic loss as an early event in AD (Masliah and Terry, 1993, Masliah, 2000). In fact synaptic loss is the best correlate of cognitive impairment in AD patients and a major contributor to cortical atrophy. The level of expression of synaptophysin in the midfrontal and inferior parietal lobes in AD patients and cognitive scores prior to death show that synaptophysin loss strongly correlates with cognitive impairment as compared to Aβ plaques or NFT (Terry et al., 1991). Synaptic loss is colocalized with neuronal loss and sometimes exceeds neuronal loss and this indicates that synaptic loss probably precedes neuronal loss. That is why synaptic density is a better correlate of cognitive
decline than neuronal loss (Terry et al., 1991, Masliah and Terry, 1993). Furthermore, it was shown that synaptic loss in AD does not happen only because of neuronal death but also in living neurons (Coleman and Yao, 2003).

In AD, the hippocampus and the frontal and parietal cortices are among the brain regions that are severely affected with synaptic loss while the other brain regions such as the occipital cortex are relatively spared (Honer, 2003). A previous study reported that synaptophysin-like immunoreactivity dramatically decreased in the hippocampus and the entorhinal cortex in autopsies of brains of patients with definite AD compared to controls or multi-infarct dementia patients (Heinonen et al., 1995).

Synaptic loss occurs later than Aβ deposition but represents a better correlate of disease progression (Terry et al., 1991, Selkoe, 2002, Shankar and Walsh, 2009). In autopsied brains of patients with moderate to severe AD, evident synaptic loss was found in the hippocampus, frontal, inferior parietal and entorhinal cortices 2-4 years after the onset of clinical symptoms. In the CA1 region of the hippocampus, synaptic loss was reported to be 18% in MCI subjects compared to 55% in mild AD subjects. Stereological investigation of the number of synapses revealed that evident synaptic loss was correlated with impaired episodic memory in MCI patients (Lu et al., 2013). Studies from animal models of AD also corroborated these findings. In the 3xTg-AD mouse model, for example, a progressive loss of dendritic spines in the somatosensory cortex was reported as early as 4 months of age in layer III neurons and it coincided with soluble Aβ and tau hyperphosphorylation at 13 months (Bittner et al., 2010). Similar data were reported in Tg2576 mice, which showed that the loss of synapses occurs in a region and age-dependent manner (Dong et al., 2007). Furthermore, brain imaging studies using
functional MRI (fMRI) showed that in AD patients global disruption of functional connectivity was associated with cognitive decline (Supekar et al., 2008).

Synaptic loss is highly correlated with the number of NFT in the same brain regions, and regional differences in the amount of synaptic loss in the cerebral cortex match the pattern of neurofibrillary degeneration as shown by BRAAK stages. A detailed study reported a decrease in the synaptophysin mRNA level in tangle-bearing neurons compared to healthy ones. However, the correlation between plaque numbers and synaptic loss was found to be less consistent and failed to establish such a relationship (Arendt, 2009).

**Synaptic compensation**

A transient increase in the level of synaptic markers at the early stages of AD as a compensation for synaptic loss is known to occur. It has been shown that the levels of synaptophysin and other synaptic proteins increase in the early stages of AD prior to NFT formation (BRAAK stage III) and then decrease again when the disease progresses, suggesting a phenomenon of synaptic compensation (Mukaetova-Ladinska et al., 2000). An increase in the level of PSD-95 in AD patients (Leuba et al., 2008a, Leuba et al., 2008b) and the presynaptic cholinergic bouton density in the midfrontal gyrus of MCI patients was reported (Bell et al., 2007). Early studies showed that synaptic loss in AD is accompanied by an increase of the synaptic size to compensate for synaptic loss. Indeed, in several neocortical areas the increase in synaptic size compensates for the significant loss of synapses by maintaining a constant total synaptic contact area. However, when the disease progresses and synaptic loss becomes more and more dramatic in these areas, the compensation phenomenon is no longer effective. The failure in synaptic compensation is more apparent in the neocortical areas that are known to be implicated early in synaptic loss such as Brodmann area 9 (Scheff and Price, 2006). These
findings were corroborated with results from fMRI studies that suggest there may be a biphasic stage in the prodromal stage of the disease in which there is a period of increased brain activation which is reduced later during the disease process (Sperling, 2007). In AD, however, although the compensatory phenomenon is present early in the disease process it becomes defective at advanced pathological stages. This phenomenon may help in slowing the cognitive deterioration seen in early AD stages and that may be why we do not see a linear relationship between brain pathology and functional measures (Arendt, 2009).

*Altered neurogenesis*

In the brain neurogenesis takes place in the subgranular zone (SGZ) of the DG and the subventricular zone (SVZ) (Fig. 4). Neurogenesis is essential for several aspects of learning and memory especially the formation of adult-born neurons is essential for pattern separation (Lacar, et al., 2014). Neurogenesis is also essential for the plasticity of the hippocampus and, especially in rodents, the olfactory system. Indeed, hippocampal neurogenesis in the CNS contributes to information storage and retrieval. Thus an impaired neurogenesis may interfere with synaptic and neuronal plasticity as well as normal neuronal function. In AD several studies reported that neurogenesis is disrupted very early in the process of the disease, with some reports claiming an increase while others claim a decrease in the level of neurogenesis. In autopsied brains an increase in neurogenesis was reported. Indeed, the expression of markers of immature neurons such as DCX, which signals the birth of new neurons, polysialylated nerve cell adhesion molecule, neurogenic differentiation factors and TUC-4 in the SVZ, granular layer of DG (site of neurogenesis) and the CA1 region (principal site of hippocampal pathology) were shown (Jin et al., 2004b). Similarly, studies employing J20 transgenic mice reported an increase in neurogenesis in the SGZ of the DG and the SVZ. Indeed, the number of BrdU positive cells and
immature neuronal marker proteins increased by 2-fold at the age of 3 months, where there was no neuronal loss or Aβ deposition in these mice. Studies on neurogenesis in human autopsied brains and transgenic mice led to the hypothesis that it is stimulated by the disease itself and not by the confounding clinical factors. The increase in neurogenesis may serve to generate new neurons to replace the degenerated ones and this may provide a unique approach to AD treatment (Jin et al., 2004a).

Neurogenesis was shown to increase after other pathological conditions such as global and focal experimental cerebral ischemia transiently and bilaterally in the SGZ and the SVZ by 12 fold, followed by normal level in 2-3 weeks. After one month it was shown that the newly born neurons migrate into the granular zone of the DG and express the calcium-binding protein (Liu et al., 1998, Jin et al., 2001, Yagita et al., 2001, Taupin, 2006). However, the aforementioned attempt of the CNS to self-repair after severe pathological insult remains unsuccessful. This may be explained in part by the fact that newly born neurons cannot differentiate into fully mature cells either because they do not develop into the right type of neurons or that they cannot integrate into the surviving circuitry. In support of this hypothesis it was previously reported that neurogenesis is unsuccessful because the newly born neurons cannot make it to become fully mature in AD brains. Indeed the levels of MAP2 a and b (markers of mature neurons) decrease dramatically in the DG of AD brains. However, the total expression of MAP2 including MAP2c (a marker of immature neurons) was less affected. These findings suggest that although in AD there is an increase in neuronal proliferation, the newborn neurons do not fully mature in the DG (Li et al., 2008). The failure of the maturation process in AD is suggested to be because of the imbalance in neurotrophic factors, including an increased level of FGF2 and decreased levels of BDNF and neurotrophin-4 (Stopa et al., 1990, Hock et al.,
2000). In fact, it was previously shown that the increase in the level of FGF-2 drives the hippocampal progenitor neurons to stay in an undifferentiated actively dividing developmental stage. Thus a promotion of the maturation of the newly born-neurons may help improve cognition and represents a potential therapeutic strategy.

Collectively, these studies then suggest that impairment in neurogenesis cannot be only the consequence of the disease but can be a cause of cognitive impairment observed in AD (Rodriguez and Verkhratsky, 2011).

The discrepancies seen among the results in the literature could be explained by the fact that neurogenesis does not follow a uniform pattern in AD and it varies between stages and between brain regions (Perry et al., 2012). The difference in several other parameters could also be involved. PS1 mutation for example, is known to have a negative effect on the generation of newborn neurons, while a non mutated PS1 would enhance cell proliferation and differentiation (Lazarov and Marr, 2010).

Fig. 4. The Process of Neurogenesis (Aimone et al., 2014).
1.4.4 Imbalance of neurotrophic factors

Neurotrophic factors are secreted molecules that play a crucial role in synaptic and neuronal growth, proliferation, maturation, pruning and survival (Budni et al., 2015). Insufficient trophic factors in the crucial regions of the brain like the hippocampus would lead to neurodegeneration and also leave them unprotected from pathological insults. The initial embryonic expression of neurotrophins approximately coincides with neurogenesis. NT-3 is mostly expressed in the immature regions of the CNS and the expression level dramatically decreases with their maturation. In contrast the expression level of BDNF is low in immature regions of the brain and it increases as these regions mature. The level of expression of NGF varies during the development of brain regions in an inconsistent manner. All three neurotrophins have a high mRNA expression level in the hippocampus, with the BDNF mRNA level 50 times higher than NGF (Hofer et al., 1990, Maisonpierre et al., 1990).

The process of adult neurogenesis generates too many newborn neurons but only a few of them survive to undergo the process of neuronal maturation and become integrated into the neuronal circuit. The selection of the neurons that will survive is based on the availability of neurotrophic factors in the target neurons. Neurotrophic factors are released by target neurons in an activity-dependent manner. A neuron that is not well connected to a target neuron would receive an insufficient amount and die, while the best connected would receive a sufficient amount and survive to become mature neurons. This ensures that the established connections and networks are synaptically active. Synaptic loss and axonal transport dysfunction are among the early events that happen in AD. The loss of synapses results in the loss of connection between the innervating neuron and the target neuron, and the axonal transport dysfunction leads to a defect in the retrograde transport of the neurotrophic factor. Both of these phenomena can lead to the death of the innervating neurons because they are not receiving the trophic factor from their
target counterpart. This is consistent with what was reported in the literature, that pro-NGF level increased in AD patients because of a defect in retrograde transport. So this suggests that neurodegeneration may be caused by disconnection between the neuron and its target rather than being a consequence of it (Castren and Tanila, 2006).

During the process of AD there is an imbalance in the level of neurotrophic factors, which may explain the failure of maturation of newborn neurons and synaptic loss. In fact, employing autopsied brains it has been reported that there is a decrease in the level of expression of BDNF in the hippocampus and the parietal cortex accompanied by a decrease in the ratio of BDNF/NT-3 in the frontal and the parietal cortices compared to control groups. However, the levels of NGF and NGF/NT-3 were significantly increased in the hippocampus and the frontal cortex in AD. The levels of NT-4/5 and NT-4/NT-3 were slightly reduced in the hippocampus and the cerebellum in the AD group compared to control cases. The level of NT-3, however, was unchanged in all the brain regions studied. The decrease of BDNF expression could then explain the lack of trophic support, which results in the degeneration of specific neuronal populations such as the cholinergic neurons. In NFT bearing neurons BDNF was not expressed, while it was expressed in tangle-free neurons, implicating its protective effect against neurodegeneration (Lanni et al., 2010).

**BDNF**

BDNF is synthesized in the entorhinal cortex and retrogradely transported to the hippocampus, where it regulates LTP and LTD, axonal sprouting, synaptic plasticity, proliferation of dendritic arbor and neuronal differentiation (Murer et al., 2001, Tyler et al., 2002). It is a vital component of synaptic plasticity and memory function. LTP provides the cellular mechanism of memory formation and consolidation and it is facilitated by BDNF
through binding to its TrkB receptor. TrkB receptors are found mainly in the hippocampus and some of them in the basic cholinergic neurons to support cholinergic function. TrKA receptors that respond to NGF are found mainly in the basal forebrain cholinergic neurons. Thus, any imbalance in the level of BDNF or NGF in the brain would affect memory formation and lead to neurodegeneration. Indeed, the level of expression of these two neurotrophins and their receptors is affected very early in AD. The level of BDNF expression was reported to be decreased by 23% in AD compared to normal controls in the frontal cortex. Pro-BDNF level also was shown to slightly progressively decrease from MCI (21%) to AD (30%) in the parietal cortex (Ferrer et al., 1999, Fahnestock et al., 2002, Michalski and Fahnestock, 2003, Peng et al., 2005, Allen et al., 2011). However, in other studies an increase in the BDNF level has been reported in the AD brain compared to MCI and control brains. TrkB levels also were reported to increase in the CA1 region of patients at early AD stage (Kao et al., 2012, Faria et al., 2014). The increase in BDNF level happened early in the disease stage and it might be as a compensation to slow down the loss of memory. However, later in the process of the disease the compensation mechanism becomes unsuccessful and the levels of BDNF are decreased dramatically. Thus, possibly for this reason the memory loss and cognitive deterioration are marked in the later stages of the disease.

Alteration in BDNF level might result in insufficient differentiation of neurons, synaptic loss and cognitive dysfunction (Song et al., 2015). Indeed, a previous study correlated the decrease in BDNF and Pro-BDNF levels to a decline in cognitive abilities very early in the disease stage (Peng et al., 2005). A recent study in the oldest old, the 90+ study, reported that the level of BDNF and Aβ 42 correlated with dementia in Brodmann areas 7 and 9. BDNF mRNA level was decreased in demented vs non-demented subjects regardless of pathology. Soluble Aβ 42 level was increased in AD subjects with or without dementia (Michalski et al., 2015).
**NGF**

NGF is synthesized in the hippocampus and it reaches its neuronal targets in the forebrain through a retrograde transport. In AD the level of NGF is reported to be decreased. However, the NGF level is unchanged or increased in the hippocampus, which argues in favor of a clear defect in the retrograde transport or an inefficient uptake of NGF to NGF-sensitive neurons. Neurodegeneration leads to an insufficient amount of NGF to reach the soma of the basal forebrain neurons, which leads to further degeneration (Hock et al., 2000, Price et al., 2007). Later studies, however, showed that the level of Pro-NGF is actually increased and the level of NGF decreased in the postmortem AD brains, and that the increase in Pro-NGF level is associated with poorer cognitive performance. The predominant form of NGF in the brain is the immature form (Pro-NGF), which is cleaved by plasmin to give the mature form (NGF). In AD the level of plasmin decreases and the Pro-NGF cannot mature into NGF. In addition, the degradation of mature NGF is increased. This is believed to be most likely a mechanism that initiates neurodegeneration of the basic forebrain neurons (Allen et al., 2011).

**FGF-2**

The brain level of FGF-2 or basic FGF is increased in AD compared to control cases and this increase is associated with the presence of neuritic plaques and NFT (Stopa et al., 1990) and with the increase in tau phosphorylation through the increase in the level of GSK3β and tau itself (Schindowskii et al., 2008). The concentration of FGF-2 is 50 times more than NGF (Siegel and Chauhan, 2000) and it is well established that FGF-2 has a promitotic role in neurogenesis. Studies on FGF receptor 1 conditional knockout mice (a major receptor of FGF-2) suggested its involvement in neurogenesis, LTP, memory consolidation but not spatial memory (Zhao et al., 2007). FGF-2 enhances adult rat hippocampal progenitor cell division and nestin level but
inhibits neuronal fate commitment and maturation of these cells in culture. FGF-2 drives the cells toward an undifferentiated actively dividing developmental stage (Chohan et al., 2011).

**GDNF**

The level of GDNF expression was reported to be increased in the cerebrospinal fluid and decreased in the sera of AD patients compared to controls, while another study showed that the level of GDNF increased in the plasma of AD patients as an adaptive compensatory mechanism from the brain (Marksteiner et al., 2011, Straten et al., 2011). However, postmortem studies showed that there is a dramatic decrease of mature GDNF in the medial temporal gyrus in AD patients. These findings were corroborated by another study that showed that the level of GDNF decreased in the sera of MCI and AD patients (Airavaara et al., 2011, Forlenza et al., 2015).

**IGF-1**

IGF-1 has been implicated in the development of late onset AD. In this context, IGF-1 was reported to have an effect on the metabolism and clearance of Aβ as well as the formation of NFT. It is even proposed that the IGF-1 receptor (IGF-1R) may promote Aβ production. Previous data also showed that the level of IGF-1R inversely correlates with cognitive impairment (Lanni et al., 2010). Indeed, a recent study reported that blocking IGF-1R signaling in adult APP/PS1 mice results in a significant delay of AD pathology through clearance of toxic Aβ (Gontier et al., 2015). In a subsequent study, however, it was reported that the levels of IGF-1R and insulin receptors were decreased in AD neurons, suggesting that neurons that undergo neurodegeneration could be resistant to IGF-1/IR (insulin receptor) signaling (Moloney et al., 2010).
1.5 Current AD therapeutics and therapeutic approaches

1.5.1 Current AD therapeutics

Currently there are six FDA-approved drugs for AD. These drugs increase neurotransmitter release and temporarily improve AD symptoms without stopping or slowing the progression of the disease. Five of these drugs are acetyl cholinesterase inhibitors. The general efficacy of cholinesterase inhibitors is considered to be moderate with 2 or 3 points on the standard cognitive outcome (Schneider et al., 2014). Based on the cholinergic theory the first cholinesterase inhibitor drug, Tacrine, was developed in 1993 but nowadays it is rarely prescribed because of possible liver damage and its low bioavailability. In 1996, Donepezil was developed and it is used for treatment of all stages of AD. Rivastigmine was developed in 2000 to treat mild to moderate stages of AD. Galantamine was approved by the FDA in 2001 to treat mild to moderate stages of the disease. In 2003, Memantine, an NMDA mild to moderate antagonist, was approved by the FDA for moderate to severe stages of AD to improve memory, attention and simple skills. However, Memantine failed to show any significant benefit when administered to patients with mild AD. From 2002-2012, out of 244 drugs tested for AD, only Memantine completed clinical trials and was approved. Late in 2014, in a second U.S-controlled clinical trial, a combination of Donepezil and Memantine (Namzaric) decreased cognitive deterioration and decline in daily activities with minimal new behavioral symptoms in AD patients when compared to placebo and was approved as a therapeutic drug for AD (Schneider et al., 2014; Alzheimer's Association 2015).

1.5.2 Anti-Aβ therapeutics

Based on the amyloid beta hypothesis several clinical trials were conducted using passive immunotherapy. Solanezumab, a monoclonal antibody against the mid-domain of Aβ, recognizes only soluble monomeric Aβ. A Phase III clinical trial using this antibody showed it to be safe but
had no improvement in primary outcome measures; Crenezumab, another antibody directed against all forms of Aβ, showed no difference between the placebo and the treatment group in the primary cognitive outcome measures; and Gantenerumab, a monoclonal antibody designed against a conformational epitope of the fibrillar Aβ, was stopped in phase II/III clinical trials because of the lack of a perceived efficacy (Rafii and Aisen, 2015).

Active immunization using a synthetic fragment of Aβ peptide named AN1792 showed exciting data in mice harboring APP mutation. Indeed, it decreased Aβ plaques and improved cognition in mice. However, when advanced to clinical trials in patients with mild to moderate AD, the active immunization was stopped because of severe side effects such as meningoencephalitis with no benefits in AD symptoms. Following AN1792 immunization, 6% of the treated patients developed meningoencephalitis. Postmortem analysis determined the cause to be an increase in CD4+ infiltration, suggesting a T-cell response to Aβ (Serrano-Pozo et al., 2011b). Based on the results of this clinical trial the National Institute on Aging (NIA) proposed that passive immunization might be a safer immunotherapeutic strategy than active immunization.

To date in clinical trials of mild to moderate AD, anti-Aβ therapy was able to clear Aβ without slowing down or stopping cognitive impairment. The repeated failures of the thousands of trials using anti-Aβ therapies are thought to be because the treatment was probably initiated too late to be effective. Indeed, it is believed that therapeutic interventions would be useless once dementia is established. Because Aβ deposits precede dementia by an average of 10-15 years, the current approach is to start treating patients during the pre-symptomatic stage and during MCI. MCI is characterized by a mild cognitive impairment without dementia, while the pre-symptomatic stage is characterized by an intact cognition that is associated with amyloid
deposition. Several large prevention studies such as the A4, DIAN, and API are being conducted targeting mainly the prodromal and the pre-symptomatic stages of AD.

Several BACE inhibitors also were tested for their therapeutic efficacy. MK-8931 is one of them. In a Phase I clinical trial this drug was well tolerated with 92% reduction of Aβ from CSF and is now undergoing Phase II/III clinical trials. Some active antibodies to Aβ also were used.

Current research now is focused mainly on developing a drug that can slow or prevent disease progression. The anti-amyloid therapeutic strategy focuses on decreasing amyloid production, inhibiting β and γ-secretases, α secretase promotors, removal of amyloid, active and passive immunization, blocking Aβ signaling and inhibiting Aβ aggregation. The anti-tau therapies focus mainly on inhibition of hyperphosphorylation, passive and active immunization and inhibition of aggregated tau. Growth factors, insulin sensitization and anti-aging drugs are being tried as therapies (Waite, 2015).

1.5.3 Tau immunotherapies

Despite the critical role that tau plays in several neurodegenerative diseases most tau-targeted drug discoveries are still in early stages and are not advanced as much as Aβ therapies. This is because the whole AD drug discovery field was focused mainly on anti-Aβ therapies due to the presence of the autosomal dominant APP mutations PS1 and PS2 in FAD cases. Several active and passive tau immunotherapeutic approaches were tested in different mouse strains and showed reduction of tau tangles and improvement in cognition (Ingelsson et al., 2004, Serrano-Pozo et al., 2011a). Different phosphotau peptides were used for active immunization while anti-tau antibodies were used for passive immunization (see Iqbal et al., 2016).
1.5.4 Prevention strategies

Based on what is commonly known — that the development of neuropathological lesions start in the brain of the presymptomatic AD patient at least a decade before developing the clinical symptoms — four large secondary prevention trials were initiated. The main goal of a secondary prevention trial is to target people who already have the neuropathological changes without the clinical symptoms and treat them to prevent progression to clearly diagnostic AD. These trials target especially high risk people such as those who carry the autosomal dominant mutations or two copies of the APOEε4 allele. The Dominantly Inherited Alzheimer’s Network (DIAN) study is testing two anti-Aβ antibodies, Solanezumab and Gantenerumab, in a cohort of people that have a mutation in PSEN-1 or PSEN-2 or APP; it is currently in a Phase II biomarker trial. The Alzheimer’s Prevention Initiative (API) trial is testing Crenezemab in the large Columbian family cohort with an autosomal dominant genetic mutation in PSEN-1. Within the next two years the API is also planning to recruit people who are homozygous to APOEε4 to test active vaccination against Aβ and BACE inhibitors. The TOMORROW trial (a clinical trial designed to delay the onset of AD) aims to test Pioglitazone to treat glucose metabolism defects in carriers of the TOMM-40 gene. The Anti-amyloid Treatment in Asymptomatic Alzheimer’s Disease (A4) study is planning to recruit people who have a high risk of cognitive impairment based on the accumulation of Aβ but with or without memory complaints. Older people aged 65-85 will be recruited in a double blind Phase III clinical trial for Solanezumab vs placebo (Sperling et al., 2014, Rafii and Aisen, 2015). However, as stated above, all the prevention trials that are initiated are mainly based on anti-Aβ therapies, while there is growing evidence now that tau spread starts even from the MCI stage, which makes it more reasonable to use a combination therapy for anti-amyloid and anti-tau in the pre-symptomatic AD patients.
1.5.5 Use of neurotrophic factors in AD treatment and the limits of their applications in clinical trials

Several neurotrophic factors such as NGF, BDNF, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and CNTF exhibit similar structure and function. Each of these factors plays an important role in neural development, maturation, proliferation, differentiation and survival of neurons. This makes the use of these factors a promising and exciting therapeutic strategy. However, their relatively big size (120Aa), short bioavailability, poor blood brain barrier (BBB) permeability and adverse side effects have led to disappointing results and limit their use in clinical trials (Saragovi and Gehring, 2000).

**NGF**

In the 1990s a small phase I clinical trial was conducted in Sweden where a mouse NGF was administered intracerebroventricularly (ICV) to three AD patients. The first two AD patients were infused with a 6.6 mg dose of mouse NGF over three months and the third patient with three doses of NGF (<0.25 mg) given separately for a total of 22 weeks. The first two patients showed slight improvement in cognitive scores while the third patient showed no improvement. However, all patients complained of lumbar pain after 11 days of treatment and two of them showed weight loss and loss of appetite. All these symptoms ceased after stopping NGF infusion (Olson et al., 1992, Eriksdotter Jonhagen et al., 1998). Another study was conducted on rats by ICV administration of a recombinant human NGF over 12 weeks. However, hyperplasia because of the proliferation of Schwann cells was a major side effect. The hyperplasia was mainly explained by the high dose of NGF infused into the ventricles and the effect was mediated through the p75NTR receptor (Winkler et al., 1997). Because of the problems encountered in the administration of NGF to the brain other means were developed and another phase I clinical
study was conducted using AD patients’ genetically modified fibroblasts with a retroviral vector to produce NGF and injection of these cells into the basal forebrain. Two of the patients were affected by the severity of the surgical intrusion and the rest showed a slowing in cognitive score deterioration by 3 points per year instead of 6 points in the MMSE score (Tuszynski et al., 2005).

Another method of NGF delivery was developed to overcome the previously mentioned problems through CERE-110; CERE-110 is a gene therapy product designed to deliver NGF to the brain through an adeno-associated virus (AAV), specifically to the nucleus Basilis of Meynert. It is administered surgically in the brain. NGF is a neurotrophic factor that enhances the survival and death of cholinergic neurons and increases acetylcholine production (Rafii and Aisen, 2015). A Phase I clinical study was successfully completed using CERE-110; it showed that CERE-110 was safe and well tolerated for two years with a long-term biologically active NGF (Rafii et al., 2014). The Phase II clinical study is currently ongoing (Mandel, 2010, Rafii and Aisen, 2015).

**BDNF**

The major limitation of the use of BDNF as a therapeutic agent for AD is that it is not biologically stable (serum half-life ≈10 min) and it cannot cross the blood brain barrier (BBB), which restricts its oral, subcutaneous or intravenous delivery. ICV injection of BDNF showed beneficial effects on memory and neurodegeneration in animal studies. However, because this approach is invasive it is used only for animal model studies and not for human clinical trials. Injecting the right dose of BDNF is of major importance because a low dose will not have any beneficial effects and a high dose will cause serious side effects (Peng et al., 2005). No human clinical trials have been conducted in AD, Parkinson’s disease or Huntington’s disease with BDNF. Five clinical trials were conducted (four with ALS and one with diabetic neuropathy) and
all of them were discouraging (Lu et al., 2013). In one amyotrophic lateral sclerosis (ALS) clinical trial, methionyl human BDNF was infused subcutaneously or intrathetically to patients. BDNF was well tolerated but it failed to show any significant effects on patients’ survival because of the small number of patients and the contradictory results. The major problem faced was that BDNF penetration was limited to the superficial layers and did not go through the parenchyma of the brain and spinal cord (Beck et al., 2005, Nagahara and Tuszynski, 2011). The most challenging problem in the use of BDNF as a drug is to deliver it directly to its target with minimal exposure to undamaged tissues.

**CNTF**

CNTF is a 22.7 KDa protein synthesized by astrocytes and Schwann cells. It is a member of the neurokine superfamily. CNTF expression develops during the second postnatal week and is almost exclusively limited to the nervous system (Hagg, 2009). In the peripheral nervous system, CNTF is synthesized in muscles and released by the motor neuron terminals and then retrogradely transported to the cell bodies. It is considered to be an autocrine and paracrine signal that is involved in the activation of astrocytes and responsible for hypertrophy in case of injury to the intact nervous system. In fact its retrograde transport increases with nerve lesion. After nerve lesion, CNTF prevents atrophy of skeletal muscles but has no effect on healthy innervated muscles, suggesting its mobilization only in a pathological state. It has been found to be decreased in the human sciatic nerve in motor neuron diseases. CNTF acts by binding primarily to the GPI-anchored alpha subunit of the CNTF receptor complex alpha (CNTFRα). This induces the recruitment of two other transmembrane receptor proteins, LIFRβ and gp130, resulting in their dimerization and tyrosine phosphorylation. This leads to the activation of the JAN kinase/STAT signaling pathway (Siegel and Chauhan, 2000). STAT proteins then
translocate to the nucleus where they bind specific DNA sequences responsible for the transcription of responsive genes (Lanni et al., 2010).

In a previous study using cultured basal forebrain cholinergic neurons isolated from 2-week-old rats CNTF was shown to act synergistically with BDNF and promote the survival of choline acetyl transferase-positive neurons. BDNF alone induced a three-fold increase of neuronal survival. However, when CNTF was added to the culture, the number of survived neurons increased to eight fold. When applied alone, CNTF has very little effect on neuronal survival. The synergetic effect was localized only to basal forebrain cholinergic neurons and not to the hippocampus, basal forebrain or cerebellum. CNTF also enhanced neuronal protection synergistically with NT-4 but not NGF. In addition BDNF mediated protection was enhanced by the LIF and not IL-6. This suggests the involvement of TrkB and CNTFR β receptors in the synergetic effects (Hashimoto et al., 1999).

When applied after motor neuron injury to animal models of neuromuscular disease, CNTF prevented neuronal death and partially restored motor function. Since CNTF is expressed mainly by glia cells in the PNS and the CNS, it has been implicated in neural stem cell fate determination and survival as well as neuronal and glia differentiation and development (Siegel and Chauhan, 2000, Lanni et al., 2010). In a recent study it has been shown that CNTF enhances forebrain neurogenesis and it is expressed in the astrocytes in the subventricular zone, the brain area where dopaminergic neurons regulate neural precursor cell proliferation. These findings suggest a link between CNTF, neurogenesis and dopamine. This link was further supported by the findings that CNTF is an endogenous regulatory component of Dopamine D2-receptor-dependent neurogenesis in the subventricular zone and the dentate gyrus in adult mice (Mori et al., 2008, Yang et al., 2008). Indeed, the dopaminergic pathway to the striatum regulates CNTF
expression and regulates neurogenesis only through CNTF (Yang et al., 2008). It was shown that CNTF supported neuronal survival in vivo and in vitro in models of neurodegeneration (Ip and Yancopoulos, 1996). However, attempts to use CNTF as a therapeutic drug were unsuccessful. Axokine, a modified version of human CNTF, with a 15 amino acid truncation at the C-terminus and substitution of two amino acids, was shown to be 5 times more potent and stable than the original CNTF in vivo and vitro (Lanni et al., 2010); when administered systematically to patients with ALS it yielded disappointing results. CNTF delivery failed to achieve sufficient concentration in the brain, with no significant efficacy; too many adverse side effects such as weight loss, muscle pain and anorexia occurred. However, despite these disappointing results CNTF clinical trials showed promising effects in Huntington disease and retinal degeneration (Lanni et al., 2010). NT-501 is a drug that consists of encapsulated human cells that are genetically modified to secrete CNTF; this drug showed significant effects in a Phase II clinical trial of retinitis pigmentosa (a photoreceptor degeneration that eventually leads to blindness) (Talcott et al., 2011).

1.6 Use of peptide mimetics to overcome the limitations of neurotrophic factor therapeutics

In neurodegenerative diseases such as AD the compensation mechanism, by increasing neurogenesis and synaptic plasticity, should at least slow down the progression of the disease at the early stages. This makes boosting neurogenesis and synaptic plasticity to treat AD a very important and exciting therapeutic approach. The design of small peptide mimetics that are biostable, able to cross the BBB and not easily degraded by peptidases can offer the opportunity to stimulate the secretion of neurotrophic factors and/or mimic their neurotrophic/neurogenic activities.
A currently approved drug, Memantine, which inhibits neuronal death, addresses only the symptoms of AD and slows down cognitive deterioration without stopping or reversing it. Since neuronal loss plays a pivotal role in cognitive impairment, one might expect that a small molecule that has a dual function of promoting neurogenesis and neuroprotection could stop the sharp and fast cognitive decline and reverse the disease state (Kelleher-Andersson, 2004). Several clinical trials were conducted using neurotrophic factors such as NGF, CNTF or BDNF as described above in sections 1.5.4 and 1.5.5. However, the major problems have been how to deliver them to the brain and how much should be delivered so that it can reach the target neurons, the severe side effects, and how the mechanism of neurotrophin signaling is integrated in the disease mechanisms (Longo and Massa, 2004). Because of the multiple effects of the neurotrophic factors on neuronal activity major side effects such as pain and gastrointestinal symptoms have been reported after BDNF delivery. More severe side effects such as fever, pain and anorexia were also reported after using CNTF in clinical trials, which limited the doses investigated (Thoenen and Sendtner, 2002). The use of small molecule neurotrophic factor mimetics can overcome the blood brain barrier permeability and stability problems encountered in the delivery of the full-length proteins.

In ligand mimetics large polypeptides are reduced to small functional units that contain the site of interaction in which the molecule can block or activate specific receptors. What makes mimetics useful is that the protein-protein interaction occurs only in a few key regions or “hot-spots” instead of the overall protein surface. Two approaches have been used to develop small peptide mimetics. The first one is to mimic antibodies for neurotrophin receptors and the second one is to mimic the ligand itself (neurotrophins). Both antibodies and neurotrophin
mimetics should bind to the same receptor as the original molecule from which they are derived (Saragovi and Gehring, 2000).

**NGF peptide mimetics**

A previous study reported the development of a promising small molecule non-peptide ligand of p75NTR that promotes survival-related signaling, inhibits Aβ induced neurodegeneration and reduces neurodegeneration in Aβ treated neuronal cultures. The molecule, named LM11-A31, is a water soluble amino acid derivative that crosses the BBB and works as an antibody analog of NGF. It has a structure similar to the NGF1 loop domain that interacts with p75NTR and it prevents neurodegeneration through the activation of the PI3 kinase/AKT signaling pathway (Longo et al., 1997). Previous work also showed that when this molecule was administrated early in the process of AD pathology right after appearance of Aβ plaques in a mouse model of AD it prevented cognitive impairment and atrophy of the basal forebrain cholinergic neurons. Administration of the molecule late in the process of AD pathology in two AD mouse models, Thy-1 hAPP Lond/Swe (APPL/S) and Tg2576, was able to reverse atrophy in the cholinergic neurons of the basal forebrain, reduced cortical atrophy and normalized the increased levels of p75NTR in the basal forebrain (Simmons et al., 2014). In another study a small molecule β-turn analog of an antibody directed against the extracellular domain of the TrkA receptor was developed and behaved pharmacologically as a partial agonist (Maliartchouk et al., 2000).

**BDNF mimetics**

The BDNF molecule has a moderate size and charge, which limits its ability to cross the BBB and its peripheral administration (Nagahara and Tuszynski, 2011). The use of a small molecule mimetic of BDNF that could be applied locally or systematically can overcome the limitations to the application of the full length BDNF molecule. It avoids the adverse side effects
of the invasive methods of delivery and uncontrolled doses, and improves BDNF diffusion (Zuccato and Cattaneo, 2009).

A small molecule that acts as a TrKB receptor agonist or modulator and could imitate the biological activity of BDNF by binding with higher affinity to TrkB and p75NTR and have neuroprotective effects through its downstream pathways would present a potential therapeutic drug for AD. One of the most remarkable BDNF mimetics that has appeared in the literature is 7,8-dihydroxyflavone (7, 8-DHF), which showed a high affinity to the TrkB receptor, imitated BDNF biological activity, reduced Aβ toxicity and synaptic dysfunction, and prevented cognitive impairment in animal models. This molecule has a high bioavailability and penetration of the BBB. However, further investigations are required to determine the stability and specificity of the drug and the agents that can interfere with its activity (Zhang et al., 2014, Song et al., 2015).

In our lab 5 different tetra peptides corresponding to different active regions of BDNF were screened. All five tetra peptides were found to be nontoxic and induce the expression of neuronal markers in mouse E18 primary hippocampal cell culture. Two of the five peptides (B3 and B5) were found to function as partial agonists/antagonists and to compete with BDNF to activate the TrkB receptor in a dose dependent manner. The primary results of this screening showed that these two peptidomimetics have neurogenic/ neurotrophic effects, can modulate BDNF signaling in a partial agonistic/antagonistic manner, and can be promising therapeutic agents for AD and other diseases where BDNF level is altered (Cardenas-Aguayo M. del et al., 2013).
**P021- a CNTF peptide mimetic**

Employing neutralizing antibodies, our lab showed previously that the neurogenic/neurotrophic effect of an anti-dementia drug, Cerebrolysin, in AD patients was due to the presence of CNTF, FGF-2, GDNF, IGF-1 and IGF-2. CNTF, however, had the strongest neurogenic/neurotrophic characteristics, and could counteract the mitogenic effect of FGF-2 and decrease the number of nestin positive cells (Chen et al., 2007). Based on these findings an epitope mapping of the neutralizing antibodies was carried out to help identify the most biologically active region of CNTF. An 11-mer peptide, peptide 6 (Aa 146-156, Ac-VGDGGLFEKKL-NH2), was discovered. Studies on C57BL6 mice showed that this peptide boosted neurogenesis by increasing the number of proliferating and differentiated hippocampal neurons. It also improved hippocampal spatial memory when administered peripherally via slow release bolus. Moreover, the peptide was found to increase synaptic plasticity by increasing in the level of expression of synaptophysin and MAP2 (Chohan et al., 2011). Peptide 6 contains a putative leukemia inhibitory factor receptor (LIFR)-binding sequence of CNTF and acts by competitively inhibiting LIF. It is BBB permeable and it has a plasma half-life of more than 6 h. In an attempt to test Peptide 6 as a potential drug in AD another study was conducted in our lab in a 3xTg-AD mouse model of AD, which harbors three mutated transgenes (APPswc, tau P301L and PS1 M146V) and shows AD like pathological features. The study was conducted on 6-7 month old animals in the mild stages of the disease process before development of any overt Aβ plaques or NFT. Peripheral administration of peptide 6 by intraperitoneal injection for one month induced neurogenesis, reduced ectopic birth in the DG, enhanced synaptic plasticity in the hippocampus and cerebral cortex and improved cognition (Blanchard et al., 2010b). In a subsequent study an experimental rat model (AAV1-2NTF-CTF infected brain) of the sporadic
form of AD was treated with peptide 6 for 40 days by daily intraperitoneal injection. Peptide 6 administration was found to rescue neurodegeneration and cognitive impairment in 2.5 month old I

2NTF-CTF rats, increasing DG neurogenesis, the mRNA levels of BDNF and synaptic plasticity as determined by the increase in synaptic and dendritic marker expression, especially in the CA1 and CA3 areas of the hippocampus (Bolognin et al., 2012). Peptide 6 was used in the Ts65Dn mouse model of Down’s syndrome. Subcutaneous administration through a slow release pellet with a continuous dosing for 30 days enhanced neurogenesis and neuronal plasticity and inhibited cognitive impairment in these mice (Blanchard et al., 2011). Furthermore, Peptide 6 treatment of neuronal cell culture and newborn Wistar rats injected with autism sera reduced neuronal death and oxidative stress and restored the balance of neurotrophic factors (Kazim et al., 2015).

The active region of Peptide 6 was further narrowed down and a four amino acid peptide (Ac-DGGL-NH2) called Peptide 6c that corresponds to the CNTF amino acids 148-151 was generated. Peptide 6c was administered subcutaneously in a continuous dose for 30 days to C57 BL6 mice. The results showed that Peptide 6c, like its parent molecule, increases neurogenesis, synaptic plasticity and cognition without weight loss or any apparent side effects such as anorexia or muscle pain. Peptide 6c acts like its parent Peptide 6 by competitively inhibiting the LIF through the JAK/STAT pathway. LIF acts by increasing self-renewal and proliferation of neural progenitor cells. By inhibiting this activity Peptide 6c would promote proliferation and maturation of neural precursor cells through CNTF signaling and thus boost neurogenesis (Blanchard et al., 2010a). Peptide 6c was adamantylated on its c-terminal to increase its lipophilicity to cross the BBB and to decrease its degradation by carboxy peptidases to make it more druggable. The pentamer Peptide-021 (P021) thus was generated (Fig. 5) with the sequence
Ac-DGGLAG-NH2. When administered peripherally to normal WT mice, P021 increased neurogenesis and neuronal plasticity, as shown by the increase in the expression of synaptophysin and synapsin1, and it significantly improved cognition by enhancing short-term memory encoding in the novel object recognition task and spatial memory in the Morris Water Maze task. P021 acts the same as peptide 6c by competitively inhibiting LIF through the slight inhibition of the LIF-induced STAT3 phosphorylation in a dose-dependent manner (Li et al., 2010).

Because aging is a great risk factor for AD, the effect of P021 was studied in a rat model of cognitive aging decline. Oral administration of P021 to 22-24 month old Fisher rats significantly reduced cognitive decline related to aging, increased neurogenesis and neuronal plasticity, increased the level of BDNF expression and significantly decreased the level of hippocampus myoinositol, a metabolite that increases with age (Bolognin et al., 2014). Recently another study was conducted in which P021 was administered in diet to 9-10 month old 3xTg-AD mice for 12 months. A significant decrease in tau pathology, probably through the increase in BDNF expression level and the inhibition of GSK3β, was found in these animals at moderate to severe stage of the disease. Furthermore, a decrease in soluble Aβ levels and a trend toward decrease in Aβ plaques load were found. P021 also increased neurogenesis and neuroplasticity and reversed cognitive impairment in these mice (Kazim et al., 2014).
Fig. 5. Steps in the design of Peptide 021 (Li et al., 2010)

1.7 The triple transgenic mouse model of AD, 3xTg-AD

The use of animal models in preclinical studies to test the effect of the drugs and their efficacy has been of great importance in advancing the drug discovery field. In AD especially several mouse models were used and they helped in better understanding molecular mechanisms of the disease and to design more efficient therapeutic approaches in attempts to cure it. The 3xTg-AD mouse model developed by Oddo et al. (Oddo et al., 2003b) harbors three mutated transgenes: APP_{Swe} mutation, tau P301L mutation and PS1 M146V knock-in. It is abiologically relevant mouse model to AD because it develops Aβ and tau pathologies in a time and topographic manner which closely mimics the human pathological distribution. Indeed, the Aβ pathology starts in the neocortex at ~6 months and expands to the hippocampus at ~12 months; NFT start at around 12 months in this model (Oddo et al., 2003a, Oddo et al., 2003b). Cognitive impairment starts as early as 3 months of age, with synaptic dysfunction preceding the onset of Aβ and tau pathologies which also mimic early cognitive impairment in AD patients. This shows the relevance of this mouse model to study AD-like pathologies compared to, for instance, the
double transgenic mouse Tg2576xJNPL3 (APP<sub>Swe</sub>), which fails to develop early behavioral impairment (Rodriguez and Verkhratsky, 2011).
Chapter 2. Materials and Methods

2.1 Animals and housing

The homozygous 3xTg-AD mice express two mutated human transgenes, APP\textsuperscript{swe} and P301L, and human PS1 M146V knock-in. Breeding pairs of these mice were obtained from Dr. Frank LaFerla through the Jackson laboratory (New Harbor, ME, USA). Wild type (WT) age-matched control from the same background strain, hybrid 129/Sv x C57BL/6, were also obtained from the Jackson Laboratory. Animals were housed and bred according to approved protocols from our Institutional Animal Care and Use Committee (IACUC), according to the PHS Policy on Human Care and Use of Laboratory Animals (revised January, 2013). Animals were housed 5 animals/cage with a 12:12 h light/dark cycle and with free access to food and water. They were given a period of acclimatization of 45 min to 1 h before any behavioral test was performed. Only young adult female 3xTg-AD mice and age-matched WT control mice were used in this study because it has been shown previously that female 3xTg-AD have more aggressive pathology than males. They show more A\textbeta deposition, worse cognitive performance and a higher deficit in neurogenesis than males (Clinton et al., 2007, Hirata-Fukae et al., 2008, Carroll et al., 2010). To exclude any effect of behavioral studies on neurogenesis, animals other than those used for behavioral studies were employed for immunohistochemical/biochemical studies (Table 1).
<table>
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Table 1. Total animals used in the study.
2.2 P021 treatment

After the Morris Water Maze task at ~ 3 months of age, the animals were divided into three groups; one group of 3xTg-AD-P021 (n= 36) treated with 60 nmole of P021/g feed in diet; another group of 3xTg-AD-vh (n = 35) treated with the same diet but without P021; and a third group of WT (n=40) treated with the same diet but without P021. P021 was formulated by Research Diets (New Brunswick, NJ). Food consumption was recorded every two weeks and body weight every month. The average mouse food consumption was \( \approx 2.7 \) g feed/day. Animals were tested behaviorally at four different time points: 0 (before treatment), 9, 15, and 16-17 months (post-treatment). 15 3xTg-AD-vh, 14 3xTg-AD P021 and 20 WT mice were used for behavioral studies. The rest of the animals were used for immunohistochemical and biochemical studies at the above time points.

2.2.1 Design and synthesis of P021

The chemical structure of P021 is shown in Fig. 6. It is a pentapeptide (Ac-DGGLAG-NH2; mol. wt. of 578.3) generated by the addition of adamantylated glycine at the C-terminus of CNTF tetrapeptide (amino acid residues 148–151). The adamantylated glycine was added to P021 to increase its BBB permeability and decrease its degradation by carboxy peptidases (Blanchard et al., 2010a, Li et al., 2010, Chohan et al., 2011). P021 was synthesized and purified by reverse phase HPLC to > 96% purity as described previously (Li et al., 2010). The sequence of the P021 was confirmed by mass spectrometry.

2.2.2 P021 stability in plasma and gastric and intestinal juices and blood–brain barrier permeability

P021 has >95% stability in artificial gastric juice during 30 minutes, ~100% stability in intestinal fluid during 120 minutes, a plasma stability of >3 hours and is BBB permeable (Kazim et al., 2014).
2.3 Behavioral Procedures
2.3.1 Elevated Plus Maze

The elevated plus maze is a test for anxiety/emotionality in mice. The test apparatus was composed of four arms (30 x 5 cm) connected together by a 5x5 cm central zone. There were two opposite facing closed arms (CA) covered by 20 cm high walls of opaque plexiglass and two opposite facing open arms (OA). The entire plus-maze was elevated on a pedestal to a height of 82 cm above floor level in a separate room from the investigator. The luminosity of the room was kept at 60 lx so that light would not exert any anxiogenic effect on the mice. Each animal was put in the center zone facing one of the open arms and it was automatically recorded for a total of 8 min using the ANY-Maze software tracking system (ANY-Maze software, version 4.5, Stoelting Co., Wood Dale, IL, USA). After each session the mouse was removed and returned to its cage. The floor was cleaned of feces with 70% alcohol to remove any olfactory cues. For each animal the time spent in CA, OA, and the total time spent in the OA + CA were recorded. Because mice prefer CA to OA, the percent of time spent in the OA was analyzed to evaluate

![Chemical Structure of P021](image_url)
anxiety-like behavior. The percentage of time in OA was calculated as the percentage of time spent in the OA over the total time spent in the OA + CA, i.e., \([\text{OA}/(\text{OA}+\text{CA})] \times 100\).

### 2.3.2 Open-field test

The open field test was used to assess anxiety-like behavior and locomotor activity in mice. An open field apparatus (a PVC square arena, 50 x 50 cm, with walls 40 cm high) was put in a separate room from the experimenter and 60 lx of light was used to avoid the anxiogenic effects of light on mice. Each animal was allowed to explore the arena for 15 min and automatically recorded using the ANY-Maze software tracking system (ANY-Maze software, version 4.5, Stoelting Co., Wood Dale, IL, USA). After each session the animal was removed and returned to its home cage. The floor was cleaned of feces using 70% alcohol to remove any olfactory cues. Movements of the animal were recorded using a camera that surmounted the open field apparatus; the camera was connected to a computer equipped with tracking software. The time spent in the central zone and the distance traveled were recorded using the ANY-Maze software. The anxiety was measured by the analysis of the percentage of time spent in the central zone and the locomotor/exploratory behavior by the total distance traveled during the 15 min duration of the test.

### 2.3.3 Accelerating Rota-rod test

Rota-rod is a test of locomotor activity in rodents. During a test trial each animal was put in one of the four chambers in the apparatus. The motor of the apparatus was set up in an accelerating mode (factory setting) at a rate of 0.02 cm/s, from 4 to 40 rpm. Each animal was put through two sessions of three trials and the fall latency was measured.
2.3.4 Prehensile traction test

This test was used to measure grip strength. The apparatus consisted of a horizontal wire 60 cm above the ground. Animals were made to grab the wire with their forepaws and then the latency to fall was recorded to a maximum of 60 s.

2.3.5 Morris Water Maze task

The Morris Water Maze task is a test of reference memory, which is a measure of hippocampal function. The test was a modification of the original procedure by Morris et al. (Morris et al., 1982). Animals were divided into three groups: 15 3xTg-AD-vh, 14 3xTg-AD-P021 and 20 age-matched wild type (WT) control mice. The procedure was performed in a circular pool tank, 180 cm in diameter. A 13 cm escape platform was submerged 1 cm below the surface of opaque water in the Northwest quadrant. The water was made opaque by the addition of a white non-toxic chalk to make the escape platform invisible to mice. Spatial cues were set up in the test room to help the animals find the platform from the distant cues. The temperature of the water was kept at 21± 1°C. In each trial the animal was given 90 s to find the escape platform. If the animal did not find the escape platform it was gently guided to it. Each animal was left for 20 s on the escape platform and then it was returned to its cage. In each trial the mouse was released from a different quadrant with its back toward the center of the tank and the starting point was changed each day so that the animal would not learn the place of the platform without using the spatial cues. The acquisition trials were conducted for four consecutive days. The retention test or probe trial, in which the escape platform was removed and the animal was allowed to swim for 60 s, was conducted 24 h after the last acquisition trial. The measure of acquisition was the time and distance swum to reach the escape platform. The measures of retention were the percent of time spent in the target quadrant and the number of entries into the target zone. Behavior of the animal in the Morris Water Maze was recorded using a Samsung digital camera (SDC 4304) mounted on the ceiling; SMART version 2.0.14 software (Pan Lab/San Diego Instruments) was used for tracking and timing of each trial.
2.3.6 Object location task

The object location task in the open field is a test for short-term spatial memory, which is mainly hippocampal dependent. In this test the 18 month old animals were required to identify the location of the object as novel or familiar based on a previous experience. The test consists of a habituation phase, a sample phase and a test phase. During the habituation phase animals underwent a total of 6 sessions, 2 sessions/day, and each session lasted 10 min within 2 h of inter-trial interval. Neophobia measurements, in which an object was put in the center of the arena and the amount of exploration time was recorded, were done during the first habituation phase. Twenty four hours after the last habituation session the sample phase and the test phase of the Novel object location were conducted. During the sample phase two identical objects were placed symmetrically in two different places, each 15 cm away from the corner, and animals were allowed to explore them for 5 min. One hour later, in the test phase, one of the two objects was moved to a new location 15 cm away from the corner, in a symmetrical position to the familiar one, and the time spent exploring each object was recorded. To evaluate cognitive performance a discrimination index was calculated as follows: \[ \text{[(time exploring the new location} - \text{time exploring familiar location)}/ \text{total time exploring both locations}] \times 100. \] Mice were considered to be impaired if they spent more time investigating the familiar location than the novel one. After each trial the objects were removed and cleaned with 70% alcohol to remove any olfactory cues.

2.3.7 Object recognition task

The novel object recognition task is a test used to evaluate short-term episodic memory, which depends on the entorhinal cortex, hippocampus and frontal cortex. In this task the 19-20 month old mice were required to identify an object as familiar or novel based on a previous
experience with one of the two objects they encountered before in the open field. The general procedure of the test consists of three different phases: a familiarization phase, a sample phase and a test phase. The familiarization phase consists of four sessions of 10 min each, one session per day for four days. During each session each animal was allowed to explore the arena for 10 min, then was put back into the cage. On the 5\textsuperscript{th} day animals were subjected to the sample phase and each two identical objects were symmetrically placed in the center of the arena 15 cm away from each corner. After 15 min delay in which the mouse was returned to its home cage, the animal was reintroduced to the arena to perform the test phase. The mouse was then presented to a familiar object (previously seen during the sample phase) and a novel object in the same location as in the sample phase. The object discrimination index was calculated as \((\text{time spent close to the new object})/(\text{time close to both old and new objects})\) during the test phase. Animals were considered to be impaired if they had a discrimination index (DI) \(\leq 0.5\). A discrimination of 0.5 means that the animal discovered both objects in a similar time and had no preference for novelty.

2.4 Tissue processing

Animals were anesthetized using an overdose of avertin and transcardially perfused using 0.1 M PBS. The brain was immediately removed from the skull. The right hemisphere was immersion fixed with 4\% paraformaldehyde in 0.1 M PBS for at least 24 h at 4\(^\circ\)C then transferred to 30\% sucrose; the left hemisphere was dissected into hippocampus, fore, mid, and hind brains, and stored at -80\(^\circ\)C for biochemical studies. After fixation and cryoprotection, the right hemisphere was sectioned into 40 um sagittal sections, then saved at -20\(^\circ\)C in antifreeze solution (ethylene glycol, glycerol and 0.1 M PBS in 3:3:4 ratio) until further processing.
2.5 Immunohistochemistry

For immunohistochemistry 3-5 animals/group were chosen randomly. Every tenth section, 5 to 6 sections/mouse, were used for staining intensity-scanning analysis. For immunofluorescence, sections recovered from the glycol antifreeze solution were washed three times, 1X PBS for 15 min then incubated in 0.5% Triton-X100 in PBS for 15 min. Sections were then washed 3 times in 1X PBS for 15 min each and blocked in 5% normal goat serum containing 0.05% Tween-20 and 0.05% Triton X-100 for 45 min. Sections were then incubated overnight at 4°C with the corresponding primary antibodies (see Table 2 for all the antibodies that were used in this study). Treatment with the primary antibody was followed by treatment with the corresponding secondary fluorescent antibody, either Alexa 488-conjugated goat anti-mouse IgG antibody (1:500, Molecular Probes, Carlsbad, CA, USA) or CY3-conjugated goat anti-rabbit antibody (1:500, Jackson Laboratory, Bar Harbor, Maine, USA), for 2 h at RT and protected from light. Sections were washed 3 times with 1X PBS for 20 min each and then mounted on microscope slides with fluorogel mounting medium (Electron microscopy, Hatfield, PA). All the slides were then covered with coverslips and stored at 4°C until analyzed by confocal microscopy under identical conditions. For all the stainings maximum projection images were generated using the average of 15 Z-stacks using the 20X objective of a Nikon 90i fluorescent microscope equipped with a Nikon C1 three-laser confocal system and a Nikon DS U1 digital camera. The entire area of the DG, CA1, CA3, parietal association and frontal cortices were analyzed. Mean pixel intensity was measured for each brain region using the Image J software package (NIH).

For Aβ staining an extra step of antigen retrieval was added right after the washes of the sections from the glycol anti-freeze solution. Briefly, 5-6 sections per mouse were put in a petri dish glass container containing 0.01 M citrate buffer and heated for 2 min until boiling. Sections
were then left to cool down at RT and treated with 50% formic acid for 5-7 min. Sections were then washed one time in 1X PBS for 15 min and then incubated in 0.5% Triton X-100 and the same procedure was followed as stated above.

To study neurogenesis the DCX and Ki67 double immunostaining were performed immunohistochemically. Primary antibodies for DCX (mouse monoclonal IgG) and Ki-67 (goat polyclonal IgG) are stated on Table 2. The corresponding secondary antibodies were used: Alexa-555 conjugated goat anti-mouse IgG antibody (1:500, Invitrogen, Camarillo, CA, USA) and Alexa-488 conjugated donkey anti-goat IgG (1:500, Invitrogen, Camarillo, CA, USA). The same procedure as stated above was used for the immunohistochemical staining with the addition of the extra step of the antigen retrieval. Sections were then counterstained with the nuclear staining TO-PRO-3 iodide (1:1000, Invitrogen, Camarillo, CA, USA) for 30 min and then washed two times for 20 min. 5 mice per group and 5 sections (every 10th section) per brain per mouse were analyzed. All sections were carefully investigated for the DCX positive staining and the Ki67 positive staining. Labeled cells in the granule cell layer and the hilus of the DG of the hippocampus were manually quantified using a 40x oil objective of a Nikon 90i fluorescent microscope. The number of DCX+ staining cells and Ki-67+ cells was reported as percent of control. DCX is a marker of immature adult-born neurons and Ki-67 is a marker of cell proliferation.

2.6 Western Blots
The brain tissue stored at -80°C from each mouse was homogenized using a Teflon-glass homogenizer to generate 10% (w/v) homogenate. The pre-chilled homogenization buffer contained 50 mM Tris.HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol plus the following protease and phosphatase inhibitors: 0.5 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 5 mM benzamidine, 20 mM β-glycerophosphate, 50 mM sodium fluoride, and 1 mM sodium orthovanadate. Protein concentration of each brain homogenate was determined by the Pierce 660 nm protein assay kit (Pierce Biotechnology, Rockford, IL USA). The tissue homogenates were boiled in Laemmli’s buffer for 5 min and then subjected to 7.5 % or 10% SDS-polyacrylamide gel electrophoresis followed by transfer of separated proteins on 0.45 µm Immobilon-P membrane (Millipore, Bedford, MA, USA). The blots were developed using the corresponding primary antibodies (Table 2). Rabbit monoclonal anti-GAPDH (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the development of the blots as a loading control. The corresponding horseradish peroxidase-conjugated affinity-pure goat anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) secondary antibodies were used (1:5,000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The blots were visualized using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA). The ECL films of the blots were scanned and then analyzed using Image J (NIH) and Multigauge softwares (Fuji Photo Film, Co., Ltd). Each protein’s band intensity quantification was the result of the subtraction of the background intensity from the original intensity of the band. Finally, the intensity of each band minus the background was normalized over the intensity of the GAPDH band minus the background. Statistical analysis was conducted using either the GraphPad Prism software package, version 5.0.
(GraphPad Software Inc., La Jolla, CA, USA), or the Stata 13 statistical package (StataCorp, College Station, TX)

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Table 2. Antibodies Used
2.7 Fluorojade C staining

Fluorojade C staining was performed according to the procedure developed by Schmued et al. (Schmued et al., 1997). Briefly, sections were mounted on positively charged slides and dried at 30-45°C in the oven for 20 min. They were briefly rinsed in distilled water followed by incubation in 100% alcohol for 3 min, 70% alcohol for 1 min, 1 min in 30% alcohol and then rinsed 1 min in distilled water. To enhance the Fluorojade C staining, sections were first incubated in 0.06% potassium permanganate (KMnO4) for 15 min (for 200 ml of distilled water, 120 mg KMnO4 was added and then stored at 4°C) then rinsed with distilled water for 1 min. A stock solution of 0.01% of Fluorojade C was prepared (25 mg of Fluorojade C powder was added to 250 ml of distilled water), then a working solution of 0.001% from the stock solution was used for the staining (20 ml of Fluorojade C stock solution and 180 ml of 0.1% acetic acid). After incubation of 30 min the sections were then washed 3 times in distilled water and dried for 20 min at 30-45°C in the oven. The dry sections were incubated 3 times in xylene for 2 min each before mounting with DPX Fluka (Milwaukee, WI). Images for the Fluorojade C positive signals were captured using a Nikon 90i fluorescent confocal microscope. The percent of area occupied was quantified using the Image J. All the pictures were taken using a 20X objective.

2.8 Statistical analysis

Statistical analyses were conducted using Stata and GraphPad Prism. Tests of scalar predictors were done with regression models; in Stata 13 (StataCorp, College Station, Tx; (Liang and Zeger, 1986, Zeger and Liang, 1986)). For multiple group comparisons one-way or two-way ANOVAs followed by post-hoc tests were used. T-tests were used for all other inter-
group comparisons. Data are presented as mean±SD. For all analyses $p < 0.05$ was considered to be significant.

### 2.9 Study design.

In order to study the synaptic compensation phenomenon which represents Specific Aim 1 of this study, 27 WT and 25 3xTg-AD-vh mice were chosen for immunohistochemical and biochemical studies. Six different age groups were used: 10, 12, 13, 14, 15, and 16 weeks. Another set of animals, 29 3xTg-AD-vh and 20 WT, went through general behavioral studies and the Morris Water Maze task at around the age of 12-14 weeks. Right after completion of the Morris Water Maze task, the mice that were used for behavioral studies were divided into three groups, 20 WT, 15 3xTg-AD-vh and 14 3xTg-AD-P021, and another set of mice that did not go through behavioral studies, 21 WT, 20 3xTg-AD-vh, and 22 3xTg-AD-P021, were employed for immunohistochemical and biochemical analyses. For Specific Aim 2 studies, the mice that were employed for behavioral analyses were used to study the effect of P021 on cognition. At 9 months after treatment with P021 the Morris Water Maze task was conducted. Then at 15-17 months after treatment the mice were tested by the object location and the object recognition tasks. For Specific Aims 3 and 4, brain samples from mice that were not tested behaviorally were taken at the age of 3 months just before the start of the treatment as a baseline measure and then at 9, 15 and 18 months after treatment with P021 (Fig. 7). P021 was given in food diet at a concentration of 60 nmol/g feed; food consumption was $\sim 2.7$/mouse/day. Food intake and body weight were measured biweekly and monthly, respectively.
Fig. 7. Study design.
IHC, immunohistochemical; 3xTg-AD-P021, P021-treated 3xTg-AD mice; 3xTg-AD-vh, vehicle diet treated mice; WT, wild type control mice.
Chapter 3. Synaptic Compensation

3.1 Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disease and the most common form of dementia in elderly people. It is characterized by two histopathological hallmarks: β-amyloidosis and neurofibrillary degeneration, maximal densities of which are seen at moderate to severe stages of dementia. However, at a very early stage synaptic dysfunction and memory loss are observed in AD. Synaptic loss is the best correlate of dementia, especially the loss of the presynaptic marker synaptophysin (Terry et al., 1991). In fact, synaptophysin immunoreactivity was reported to decrease by 25% in the cortex of mild AD patients compared to normal cases (Masliah et al., 1995, Masliah et al., 2001). Degeneration of the whole synaptic element, seen as simultaneous loss of presynaptic proteins synaptophysin and Rab 3A and postsynaptic protein synaptopodin, was found in the cortex of AD patients (Davidsson and Blennow, 1998). All laminas of the neocortex in AD patients suffer from a synaptic loss of approximately 50% (Masliah et al., 1989) and the quantification of the neuronal and synaptic density in the temporal and frontal cortices in AD showed a decrease of 48% in the synapse to neuron ratio (Bertoni-Freddari et al., 1996).

The use of transgenic animal models of AD made it convenient to understand the progression of AD pathology and to develop treatments. Herein, I used the 3xTg-AD mouse (Oddo et al., 2003b), a widely used model shown to mimic the neuropathological features of AD such as Aβ and tau pathologies. The Aβ pathology starts at around nine months of age, tau pathology at around twelve months of age and cognitive impairment as early as three months of age in this mouse model (Davis et al., 2013). This model was generated by the co-injection of
two mutated human transgenes, APPswe (KM670/671NL) and tau P301L, into the PS1/M146V knock-in embryo (Oddo et al., 2003b). The two mutated transgenes are under the control of Thymine 1.2 promoter, which is activated during the embryonic days 11 and 15 in mice (Campsall et al., 2002).

In the present study I show that in a manner reminiscent of AD, in 3xTg-AD mice cognitive impairment is associated with a transient synaptic compensation, which is followed by synaptic deficit.

3.2 Results

3.2.1 At three months the 3xTg-AD mice are impaired in spatial reference memory

Since AD is characterized by loss of short-term memory in the early stages of the disease as well as loss of long-term memory at later stages, I investigated if young adult 3xTg-AD mice are cognitively impaired. Because the hippocampus is the main brain structure implicated in memory formation, storage, consolidation and retrieval I employed the Morris Water Maze behavioral task, which is hippocampal-dependent, to test 3xTg-AD mice. In this task, mice are required to learn the position of a hidden platform using distal spatial reference cues. At the end of the learning trials mice have to form a spatial navigation map using the spatial cues to determine the position of the platform.

The analysis of the swim speed revealed no significant difference between the wild type and the 3xTg-AD mice (Fig. 8a). For this reason, the learning performance of the animals was analyzed as the latency time to reach the escape platform. I found that animals from the two different groups learned the task well. This was evident from the decrease in the latency to escape across training sessions. However, when I compared the escape latency curve across time, I found that the 3xTg-AD learned slower than the WT across the training days (Fig. 8b, p =
These data revealed that at 12-14 weeks 3xTg-AD mice were impaired in spatial reference memory.

To evaluate the memory retention in the 3xTg-AD and WT mice, a probe trial 24 h after the last training session was conducted. The 3xTg-AD mice spent significantly less time in the target quadrant (Fig. 8c; p<0.001) and visited the target zone less (Fig. 8d; p<0.005) than WT animals. These results suggest that memory retention was compromised in 3xTg-AD mice and further corroborate our findings that these mice were cognitively impaired.
Fig. 8: At 12-14 weeks the 3xTg-AD (Tg-AD) mice show spatial reference memory impairment in Morris Water Maze test when compared with control WT animals.
a) Swim speed, b) Escape latency, c) Percent of time in target quadrant, d) Number of platform crossings. *p < 0.05; **p < 0.01; ***p < 0.001. (WT, n = 20; Tg-AD, n = 29). Data are shown as mean ± SD.

3.2.2 3xTg-AD mice have higher locomotivity and motor coordination than WT mice

The Rota-rod and prehensile traction tests were employed to evaluate locomotivity and motor coordination. The 3xTg-AD mice had significantly higher latency time to fall in the Rota-rod test (Fig. 9a-b; p<0.001) and prehensile traction test (Fig. 9c; p<0.001) than WT. Furthermore, the difference increased over trials, showing that the 3xTg-AD mice had enhanced motor coordination and motor learning. Similar findings were reported previously (Blanchard et al., 2010b, Oore et al., 2013, Stover et al., 2015).

3.2.3 Anxiety level of 3xTg-AD mice is similar to WT mice

In the Elevated Plus Maze task the open arm is considered to be more anxiogenic than the closed arm. Although I found previously that at 6 to 9 months of age the 3xTg-AD mice show more anxiety than WT, in the present study the 12-14 week old 3xTg-AD mice did not show any increase in anxiety compared to WT in the Elevated Plus Maze (Fig. 9d).

I also measured the level of anxiety by open field test in 3xTg-AD and WT mice. Animals are considered to be anxious if they spend less time in the center than in the periphery of the arena. There was no significant difference between the two groups in the distance covered during exploration or in time spent in the center zone (Fig. 9e-f).
Fig. 9: At 10-12 weeks of age the Tg mice had markedly higher locomotor activity than WT controls.

a) Rota-rod, b) Prehensile traction test, c) Elevated plus maze, d) Open field. *p < 0.05; **p < 0.01; ***p < 0.001. (WT, n = 20; Tg-AD, n = 29). Data are shown as mean ± SD.
3.2.4 Impairment in spatial reference memory is accompanied by an increase in neuronal and synaptic markers to compensate for synaptic loss in 3xTg-AD mice

Synaptic loss is an established phenomenon and an early event in AD. Synaptophysin loss in the frontal cortex strongly correlates with dementia and cognitive impairment (Masliah et al., 2001). To examine synaptic loss at very early stages of the disease in different brain regions, 3xTg-AD mice aged 10, 12, 13, 14, 15, and 16 weeks were studied. Brain sections from these animals were immunohistochemically stained using four different antibodies: anti-synaptophysin, anti-GluR1, anti-MAP2, and anti-beta-III tubulin.

3.2.5 CA1 region

Longitudinal changes in mean pixel intensity showed that the synaptophysin expression in 3xTg-AD mice from 10-16 weeks of age was best modeled by a cubic regression model. In this model synaptophysin expression diminished at 12 weeks of age and was then compensated for from 13 to 16 weeks of age (Fig. 10a; Table 3). Similarly, Western blots of synaptophysin expression were best fit by a cubic model, with a significant decrease in synaptophysin level in the hippocampus at 12 weeks, followed by an increase by 13-14 weeks and then a decrease by 15-16 weeks in 3xTg-AD mice (Fig. 11a-b, F (3, 18) = 5.47, p = 0.0075).

The examination of GluR1 expression in the CA1 region showed no significant difference across time (Fig. 10b). Similarly, Western blots showed no change of the expression level in the hippocampus.

The level of expression of the dendritic marker MAP2 was best fit by a linear model, though the actual level of MAP2 decreased at 12 weeks of age and then increased from 13-16 weeks (Fig. 10c; Table 3).
The level of expression of β-III tubulin dropped at 12 weeks and then increased from 13-16 weeks (Fig. 10d; Table 3), though again the best fit was obtained with a linear model.

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Table 3. Longitudinal best-fitting regression models for immunohistochemistry of Tg-AD across time.
Fig. 10: In the CA1 region, Tg mice showed a decrease in synaptophysin, MAP2 and β-III tubulin at 12 weeks which was compensated by 13 weeks.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD.
Fig. 11: Expression levels of synaptophysin and GluR1 in the hippocampus in Western blots.
Longitudinal analysis of synaptophysin (10-16 weeks). (a, b) and (c, d) cross-sectional (10-13 weeks, Tg and WT mice). a, c are Western blots. *p<0.05; **p<0.01; ***p<0.001. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group).

3.2.6 CA3 region

The level of expression of synaptophysin in the CA3 region was best fit by a cubic model (Fig. 12a; Table 3). As in the CA1 region, the expression of synaptophysin dropped at 12 weeks and increased from 13 to 16 weeks of age. No significant change was detected in the GluR1 and MAP2 levels in the CA3 region (Fig. 12b, c; Table 3). β-III tubulin was best modeled by a cubic regression with a decrease in the level of expression at 12 weeks and increase from 13-16 weeks of age (Fig. 12d; Table 3).
Fig. 12: Tg mice showed a decrease in the level synaptophysin and β-III tubulin in the CA3 region at 12 weeks, followed by a compensatory increase by 13 weeks.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD.
3.2.7 DG region

In the DG the level of synaptophysin dropped at 12 weeks of age and then increased from 13 to 16 weeks. The changes followed a cubic model (Fig. 13a; Table 3). No change was found in the expression of GluR1 (Fig. 13b). The level of expression of MAP2 followed a quadratic model (Fig. 13c, Table 3); β-III tubulin expression was best fit by a quadratic model with a drop at 12 weeks and increase from 13-16 weeks (Fig. 13d; Table 3).
Fig. 13: Synaptophysin, MAP2 and β-III tubulin in the DG region decreased at 12 weeks for synaptophysin and 13 weeks for MAP2 and β-III tubulin with compensatory increase at later ages.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD.
3.2.8 Parietal cortex region

Expression of synaptophysin, MAP2, and β-III tubulin showed a drop in the expression level across time at 12 weeks and an increase from 13-16 weeks. A cubic model best fit the expression of synaptophysin, while β-III tubulin and MAP2 followed a quadratic model (Fig. 14a, c, d; Table 3). The level of expression of GluR1 did not change significantly (Fig. 14b; Table 3).
Fig. 14: The levels of synaptophysin, β-III tubulin and MAP2 in the parietal cortex showed a decrease at 12 weeks, followed by a compensatory increase by 13-16 weeks.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. Scale bar: 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD.
3.2.9 Frontal cortex region

The level of expression of synaptophysin and MAP2 changed across time. Synaptophysin followed a cubic model (Fig. 15a; Table 3) while for β-III tubulin a linear model fit best (Fig. 15d; Table 3). Both markers decreased at 12 weeks of age and increased from 13-16 weeks. No significant change was detected in the expression of GluR1 (Fig. 15b; Table 3) or MAP2 (Fig. 15c; Table 3).
Fig. 15: In the Frontal cortex, the levels of synaptophysin and β-III tubulin significantly changed at 12 weeks. No change was detected for the GluR1 level. Compensation happened from 13-16 weeks.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. Scale bar: 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group ). Quantification is shown as mean ± SD.

3.3 Cross-sectional comparison between 3xTg-AD and control mice confirms synaptic compensation in the former

3.3.1 CA1 region

Cross-sectional comparison of 3xTg-AD with WT mice revealed a comparative synaptic loss at 12 weeks followed by compensation in different brain regions at different ages. In the CA1 region there was a non-significant trend toward decrease in synaptophysin expression at 12 weeks and a relative increase from 13 to 15 weeks (Fig. 16a; Table 4). Western blots showed changes in synaptophysin expression best fit by a quadratic model with synaptic loss at 12 weeks compensated from 13 weeks (Fig. 11c-d, F (2, 7) = 12.56; p < 0.005).

GluR1 non-significantly increased in 3xTg-AD mice compared to WT at 13 weeks of age, although there was no decrease at 12 weeks (Fig. 16b; Table 4).

Similarly, MAP2 expression was comparable in 3xTg-AD and WT mice, though the level of MAP2 decreased at 13 weeks, increasing at 14 weeks (Fig. 16c; Table 4).

β-III tubulin did not change significantly but the lowest level was at 13 weeks of age with a slight compensation from 14-16 weeks of age (Fig. 16d; Table 4).
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Table 4: The cross-sectional analysis of WT vs Tg-AD immunohistochemically across time.
Fig. 16: Cross-sectional comparison of Tg and WT mice for the relative expression of synaptophysin, GluR1, MAP2 and β-III tubulin in the CA1 region from 12 to 16 weeks showed a decrease at 12-13 weeks, followed by compensation at different ages in different brain regions in Tg mice.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 25; n = 3 to 5/group). Quantification is shown as mean ± SD. This experiment was repeated 3 times.
3.3.2 CA3 region

Synaptophysin expression did not differ significantly between 3xTg-AD and WT. The lowest level of synaptophysin was detected at 12 weeks in 3xTg-AD mice with an increase at 14 weeks (Fig. 17a; Table 4).

GluR1 expression did not change significantly, decreasing at 13 weeks and increasing only at 14 weeks (Fig. 17b; Table 4).

MAP2 decreased at 12 weeks, increased at 15 weeks, then dropped significantly at 16 weeks (Fig. 17c; Table 4).

β-III tubulin expression in the CA3 did not change significantly. The lowest level was at 13 weeks of age with a slight increase from 14-15 weeks and a decrease at 16 weeks (Fig. 17d; Table 4).
Fig. 17: Cross-sectional comparison of Tg and WT mice for relative expression of all the markers in the CA3 region from 12 to 16 weeks showed a non-significant decrease in the MAP2 level at 12 weeks followed by a compensation and then a decrease again at 16 weeks. No significant changes were found for the other three markers. a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD. This experiment was repeated 3 times.
3.3.3 DG region

Synaptophysin in the DG did not differ significantly between WT and Tg-AD across time, though some compensation occurred at 15 weeks (Fig. 18a; Table 4).

Overall there was no significant difference in the expression of GluR1; it was lowest at 12 and 13 weeks followed by compensation at 15 weeks (Fig. 18b; Table 4).

MAP2 decreased at 12 weeks, increased from 13-15 weeks, and then dropped again at 16 weeks (Fig. 18c; Table 4).

Overall β-III tubulin did not change significantly. The lowest level was at 13 weeks with an increase at 15 weeks (Fig. 18d; Table 4).
Fig. 18: Cross-sectional comparison of Tg and WT mice for relative expression of synaptophysin, GluR1, MAP2 and β-III tubulin from 12 to 16 weeks in the DG region showed a non-significant decrease for the MAP2 level at 12 weeks, a compensation from 13-15 weeks and a decrease again at 16 weeks. No significant changes for the other three markers occurred. a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals. *p<0.05; **p<0.01; ***p<0.001. Scale bar: 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD. This experiment was repeated 3 times.
3.3.4 Parietal cortex region

Synaptophysin decreased significantly at 12 weeks and increased from 13-16 weeks (Fig. 19a; Table 4).

GluR1 did not differ significantly; the lowest level was at 12 weeks with an increase at 14 weeks (Fig. 19b; Table 4).

MAP2 change was not significant; the lowest level was at 13 weeks with an increase at 14 weeks (Fig. 19c, Table 4).

β-III tubulin was lowest at 13 weeks and increased at 14 weeks (Fig. 19d. Table 4). The changes were not significant.
**Fig. 19**: Cross-sectional comparison of Tg and WT mice for relative expression of synaptophysin, GluR1, MAP2, and βIII tubulin from 12 to 16 weeks in the parietal cortex. Only synaptophysin showed a decrease at 12 weeks followed by compensation at later time points.

a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD. This experiment was repeated 3 times.

### 3.3.5 Frontal cortex

Synaptophysin decreased at 12 weeks and increased at 14 weeks. The compensation for synaptic loss occurred from 13 to 15 weeks (Fig. 20a; Table 4).

GluR1 non-significantly decreased at 13 weeks and rose from 14-16 weeks (Fig. 20b; Table 4).

MAP2 did not change significantly. The lowest level was at 13 weeks, rising at 14 weeks (Fig. 20c, Table 4).

β-III tubulin in Tg-AD mice was lowest at 12 weeks and increased non-significantly at 14 weeks of age. Compensation for synaptic loss happened mainly at 14 weeks (Fig. 20d; Table 4).
Fig. 20: Cross-sectional comparison of Tg and WT mice for relative expression of synaptophysin, GluR1, MAP2, and βIII tubulin from 12 to 16 weeks of age in the frontal cortex.

The levels of synaptophysin, MAP2, and β-III tubulin decreased from 12-13 weeks and then increased at 14-16 weeks. a) synaptophysin; b) GluR1; c) MAP2; and d) β-III tubulin. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals.

* p<0.05; ** p<0.01; *** p<0.001. Scale bar = 100 µm. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD. This experiment was repeated 3 times.
3.4 Changes in expression levels of neuronal and synaptic proteins in WT mice

3.4.1 CA1 region

Synaptophysin and GluR1 did not change significantly in WT mice (Fig. 21a; Table 5). MAP2 and β-III tubulin significantly changed over time in the CA1 region, best modeled with a cubic regression (Fig. 21a; Table 5). MAP2 decreased from 12 to 13 weeks and came back to normal level at 14 to 15 weeks. β-III tubulin decreased at 12 weeks and then increased at 13 weeks.

3.4.2 CA3 region

No significant changes were detected in synaptophysin, MAP2 or β-III tubulin (Fig. 21b; Table 5). GluR1 followed a cubic pattern, with the lowest level at 15 weeks and an increase at 16 weeks (Fig. 21b; Table 5).

3.4.3 DG region

No significant changes for any of the markers were detected in the DG area (Fig. 21c; Table 5).

3.4.4 Parietal cortex

The only change that was seen in this region was in the expression level of GluR1. It followed a quadratic fit with a decrease from 12-15 weeks and then an increase by 16 weeks (Fig. 21d; Table 5). All the other markers showed no differences.

3.4.5 Frontal cortex

No significant change was seen for any markers in this brain region (Fig. 21e; Table 5).
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Table 5: Longitudinal comparison of WT immunohistochemically across time.
Fig. 21: Longitudinal comparison of relative expression of synaptophysin, GluR1, MAP2 and βIII tubulin in WT mice showed some changes but no overall decrease from age 10 weeks to 16 weeks.

a) CA1; b) CA3; c) DG; d) parietal cortex and frontal cortex. Sections were immunostained and analyzed by confocal microscopy in a single experiment under identical conditions and settings so that they could be directly compared across the six age groups. *p<0.05; **p<0.01; ***p<0.001. (WT, n = 28; Tg-AD, n = 26, n = 3 to 5/group). Quantification is shown as mean ± SD.
Cross-sectional comparison between 3xTg-AD and control mice shows that synaptic compensation happened at different time points in different brain regions.

The comparison of the changes in the expression of the different synaptic and neuronal markers mentioned above in the 3xTg-AD mice as % of WT showed that synaptic compensation happened in different brain regions at different time points (Fig. 22, table 5). Indeed, for the synaptic marker synaptophysin for example synaptic compensation in the CA3 region happened at 14 weeks (Fig. 22, table), while in the parietal cortex compensation happened at 13 weeks (Fig. 22, table 5). Table 5 shows all the regional changes for all the synaptic and neuronal markers at different time points.
Fig. 22: Change in Tg mice as % of WT showed that synaptic compensation occurred at different time points in different brain regions. A summary of data taken from Figures 16-20. Sections from WT and Tg mice at each age were immunostained and analyzed by confocal microscopy under identical conditions and settings to enable comparison between the two groups of animals. WT (n = 28, 3xTg-AD, n = 26, n = 3 to 5/group).
3.5 Discussion

Dementia status in AD is preceded by a relatively long and variable period of MCI. In some individuals the MCI stage does not progress to AD. This variable MCI stage is probably due to the compensatory neuroregenerative response of the AD brain which, because of an insufficient neurotrophic environment, does not succeed completely (Li et al., 2008, Iqbal et al., 2014a). Histopathological studies have shown that the compensatory phenomenon does occur in the AD brain (Mukaetova-Ladinska et al., 2000).

The present study revealed that the spatial reference memory impairment in the young adult 3xTg-AD mice seen at around 12-14 weeks of age is accompanied by a decrease in the presynaptic marker synaptophysin, the postsynaptic marker GluR1, the dendritic marker MAP2 and the neuronal marker β-III tubulin. However, reminiscent of the AD brain, the 3xTg mice showed a decrease in synaptic and neuronal markers at 12 weeks followed by a transient increase from 13 to 16 weeks of age. I speculate that this increase is a failed attempt of the brain to compensate for synaptic loss. Beyond the age of 4 months, the 3xTg-AD mice are known to have a decrease in synaptophysin as well as an apparent synaptic dysfunction (Oddo et al., 2003b, Blanchard et al., 2010b). Most importantly, when I expressed the data of 3xTg-AD mice as a percent of control (WT) for different ages from age 10-16 weeks, different brain regions compensated for the synaptic loss at different time points. Although the WT mice also showed changes in these markers from 10-16 weeks of age, the changes seen in WT mice were totally different from those seen in 3xTg-AD mice.

The present study showed that there was an upregulation in the level of expression of different synaptic, dendritic and neuronal markers, especially the increase in the expression level of synaptophysin in cognitively impaired 3xTg-AD mice. Previous studies reported an increase
in glutamatergic buttons in the midfrontal gyrus in MCI patients and an increase in synaptophysin immunoreactivity and cholinergic buttons in the plaque proximity areas in the APP \textsubscript{K670N,M671L} \textsubscript{+} PSI\textsubscript{M146L} double transgenic mice which, however, decreased with the progression of the disease (Hu et al., 2003, Bell and Cuello, 2006, Bell et al., 2007). Similarly, spatial reference memory impairment in aged rats was shown to be associated with an increase in the levels of expression of PSD95 and GluR1 compared to control animals (Nyffeler et al., 2007).

Consistent with the present study, a previous study reported an increase in the level of expression of synaptophysin and SNAP-23 in AD brains at Braak stage 3 with minimal to mild clinical grades of dementia compared to normal controls and an increase in the expression levels of MAP2 and \(\alpha\)-synuclein at Braak stage 4 in the neocortex in AD (Mukaetova-Ladinska et al., 2000). However, a sharp decrease in synaptophysin expression in AD was seen only at Braak stages 5 to 6 with the full spectrum of amyloid beta and tau pathologies and moderate to severe clinical grades of dementia. Similarly, an increase in the level of PSD-95 expression in the entorhinal cortex of AD cases (Leuba et al., 2008a; Leuba et al., 2008b) and a decrease in the level of synaptophysin expression compared to age-matched controls was previously reported (Leuba et al., 2008b). The agreement between human and animal studies indicates that the upregulation in synaptic, dendritic and neuronal markers found in the present study is most probably due to a compensatory phenomenon initiated by the diseased brain to save the remaining intact synapses.

In light of what was found previously in human subjects, findings of the present study in 3xTg-AD mice could be mimicking what happens in AD patients at early stages of the disease. For instance, at the age of 12-14 weeks the 3xTg-AD mice are considered to be in the early
stages of AD-like disease. They have only intracellular amyloid beta (Aβ) and cognitive impairment; Aβ plaque and tau pathologies are seen only at 9-12 months onwards.

It is believed that synaptic compensation could happen at different levels: at the activity level, at the network level and at the synaptic level. At the activity level compensation occurs when a neuron senses its own spiking activity level through calcium-dependent sensors and adjusts it accordingly by the phenomenon of synaptic scaling, mainly through adjusting the abundance of glutamate receptors postsynaptically. The neuron can also self-adjust by local synaptic compensation through a change in the efficacy of neurotransmitter release presynaptically. At the level of the neuronal network, when target neurons are denervated they inform afferent neurons via signaling about the need for synaptic input. Remaining intact neurons compensate by increasing the synaptic area so that the total synaptic contact area per unit volume of the cortex remains intact (Scheff, 2003).

Coupling between astrocytes and neurons can also contribute to global synaptic homeostasis because glial cells are well placed to sense the overall activity of neurons (Stellwagen and Malenka, 2006). Using random synaptic deletions to simulate global synaptic compensation, computational studies showed that all the remaining intact synapses would increase their activity (or scaling) in a similar way by multiplying their weight by the same factor (Horn et al., 1993). From a neurobiological perspective, synaptic compensation could happen by the upregulation of the expression levels of certain presynaptic and postsynaptic markers, e.g., the increase in the expression levels of synaptophysin, PSD95 and GluR1 reported previously (Leuba et al., 2008a, Leuba et al., 2008b, Hu et al., 2003, Nyffeler et al., 2007) as well as the findings of the present study. The synaptic compensation could result from neuritic outgrowth. Indeed, it has been proposed that after their death affected neurons liberate growth factors that
could be used by surviving neurons for growing new neurites (Uylings and de Brabander, 2002). Another mechanism of synaptic compensation could be activation of silenced synapses following the death of inhibitory neurons and an increase in neurogenesis in the dentate gyrus (Jin et al., 2004b).

There are three major points of view concerning the mechanism of synaptic compensation. One view considers the phenomenon to be beneficial, helping to slow down the progression of the disease (Iacono et al., 2008). The second view considers it as detrimental because it affects the brain network and drives disease progression (Nyffeler et al., 2007, Abuhassan et al., 2014, Berchtold et al., 2014). The third one considers compensation as beneficial at the early stage of the disease because it slows down its progression (Mesulam, 2000, Small, 2008). However, in the long run it is detrimental because it becomes the driving force to affecting the whole neuronal network.

I speculate that in the present study the decrease in synaptophysin, MAP2 and β-III tubulin at 12 weeks of age in the 3xTg-AD animals compared to WT and the transient increase in these three markers from 13 to 16 weeks of age is due to a compensatory mechanism in which the diseased brain attempts to repair itself. Because the disease is not fully developed in the young 3xTg-AD mice, the brain still has the capacity to compensate for the synaptic loss. This compensatory mechanism may be the biological basis of slowing down the development of the AD-like pathological features in this mouse model. Indeed, it is known that the 3xTg-AD mouse develops Aβ and tau pathologies only at an advanced age: Aβ pathology at ~9 months and tau pathology at around 12 months. Thus, it takes several months for these pathological features to develop and this is considered a long enough period for the progression of the pathologies. I speculate that a similar phenomenon may be occurring in asymptomatic AD or MCI patients’
brains. In fact, the mild cognitive impairment period is relatively long, lasting several years, and this may be due to the compensation or the self-repair attempt of the brain. However, as the disease progresses the brain is overwhelmed and synaptic loss reaches a certain threshold level beyond which the brain cannot compensate any more. Perhaps this is why the acceleration of cognitive decline and the appearance of clinical features of AD occur at an advanced stage.

For this reason, prevention strategies starting at an early stage of the disease could be more effective than intervention at a later stage after a drastic synaptic and neuronal loss is reached. Once the brain has undergone a severe insult of synaptic and neuronal loss, repair would be more difficult than prevention at an early stage of the disease. Acting when synaptic compensation is most active would likely be more effective.
Chapter 4. Effect of early treatment with P021 on cognitive impairment

4.1 Introduction

The cumulative incidence of AD is estimated to increase from ~5% at the age of 70 to ~50% at the age of 90 (Jahn, 2013). AD interferes with the process of memory formation from the molecular level to the level of the whole brain network. In most cases of AD, slow cognitive deterioration occurs several years before the clinical symptoms are brought to attention. During the very early stages of AD, short-term memories are severely affected and patients have difficulties remembering new information and familiar people’s names. Executive functions such as judgment and problem solving and organizational skills are also affected; long-term and declarative memories, however, are less deteriorated. Small personality and behavioral changes are reported. Agitation, anxiety and psychosis are not reported in the initial stages. In contrast, during moderate to severe stages cognitive deterioration becomes more pronounced and newly learned material is rapidly lost with patients described as “living in the past”. Difficulties in spatial navigation and disorientation in the familiar environment become marked symptoms as well as the inability to recognize family members and close relatives. Executive function and logical reasoning are severely deteriorated while behavioral symptoms such as hallucinations, delusions and illusionary misidentification become more common. Sleep disruption, agitation with temper tantrums, physical and verbal aggression and anxiety are also common in this stage. Apathy, however, is the most persistent and frequent symptom exhibited by 72% of the patients through all stages of AD (Mega et al., 1996). Behavioral disturbances represent the major burden and source of distress to the caregiver and lead to elder abuse in the US. This results in the
institutionalization of patients to nursing homes, which leads to an increase in the financial cost (Binetti et al., 1998). At the most severe stages almost all the cognitive functions are gone, with patients becoming totally dependent on caregivers and having difficulties even in chewing and swallowing food. Most patients become bedridden at this stage and the major cause of death is pneumonia (Lyketsos et al., 2011, Tarawneh and Holtzman, 2012). Women are known to be at a higher risk than men to suffer from AD. This may be because of the decrease in the level of estrogen after menopause (Irvine et al., 2012).

The severity of cognitive impairment directly correlates with the density of NFT. NFT formation starts in the memory-associated areas such as the entorhinal cortex and the hippocampus, then spreads to parietal, temporal and finally frontal cortices. Indeed, findings from the Nun Study and the Adult Changes in Thought (ACT) study reported a strong correlation between the BRAAK stages and the status of dementia (SantaCruz et al., 2011). Furthermore, the NFT density in the entorhinal cortex and the temporal lobe showed a strong correlation with cognitive scores in non-demented people. Another study also suggested that the accumulation of NFT may be responsible for memory loss associated with normal aging as well as the memory deficit seen in MCI (Guillozet et al., 2003). In contrast, Aβ plaque deposition did not correlate either with neuronal loss and NFT or with the severity of the disease (Gomez-Isla et al., 1997). The number of senile plaques in the cerebral cortex also tends to stay stable with the severity of the disease stages. The fact that Aβ plaque deposition does not correlate with cognitive scores, is not specific for memory related lesions and is found in many cognitively normal individuals (Tomlinson et al., 1970, Alafuzoff et al., 1987, Arriagada et al., 1992b, Guillozet et al., 2003) strongly suggests that Aβ burden does not play a significant role in memory impairment during the early stages of the disease (Guillozet et al., 2003). However, the spatial and temporal
distribution of the NFT and the numerical density better match the clinical features of dementia than the Aβ plaque burden (Tomlinson, 1982, Alafuzoff et al., 1987, Arriagada et al., 1992a, Berg et al., 1993, Gomez-Isla et al., 1997, Berg et al., 1998, Giannakopoulos et al., 2003). The severity of cognitive symptoms was found to be even more correlated with the severity of neuronal loss (Gomez-Isla et al., 1997) and even more with synaptic deficit, especially with synaptophysin loss (DeKosky and Scheff, 1990, Terry et al., 1991).

Medical intervention delaying disease onset by 5 years could reduce the number of AD patients by 57% and decrease the projected Medicare financial costs from $627 billion to $344 billion (Sperling et al., 2011). Once a reliable screening instrument is developed and a treatment that slows the progression of the disease by 50% is discovered, the risk of having AD in people aged 65 and older would decrease from 10% to 5.7%. Furthermore, even delaying the onset of the disease by 6 months would, it is estimated, decrease the number of people developing AD by half a million by 2050 (Brookmeyer et al., 1998). Currently approved drugs produce only modest improvements of cognitive symptoms, resulting mostly in stabilization rather than alleviation of the cognitive symptoms (O'Hara et al., 2001).

The use of transgenic mouse models greatly helped to understand the molecular mechanisms of AD and facilitated preclinical studies that could lead to development of potential AD drugs. The 3xTg-AD mouse model has proven to be a biologically relevant model to AD pathology. Unlike other transgenic mice, 3xTg-AD mice develop Aβ plaques and hyperphosphorylated tau without NFT, resembling rarer cases of AD (Tiraboschi et al., 2004). It is even recently reported that these mice develop a deficiency in episodic memory as early as 3 months of age that deteriorates similarly to the deterioration seen in patients. In the present study I found (Chapter 3) that, similar to the visuospatial impairment seen in AD, the 3xTg-AD mice
are impaired in spatial memory as examined by the Morris Water Maze task. The aim of this study is to investigate the effect of P021 on preventing or delaying the cognitive impairment by initiating the treatment at 3 months, which is around the period of synaptic compensation. P021 and its parent molecule Peptide 6 were previously shown to be effective in reversing cognitive impairment in 3xTg-AD mice when the treatment was started at later stages of the disease (Blanchard et al., 2010b, Kazim et al., 2014). P021 was also found to rescue cognitive impairment in aged Fisher rats (Bolognin et al., 2014) and in a rat model of the sporadic form of AD (Bolognin et al., 2012).

In the present study, I investigated the effect of P021 treatment starting at around the period of synaptic compensation and lasting for ~18 months in 3xTg-AD mice. I found that, reminiscent of AD, cognitive deterioration in 3xTg-AD mice starts very early in the disease process and lasts for the lifetime. Furthermore, P021 treatment right after the onset of cognitive impairment and very early in the disease process can completely prevent cognitive impairment in 3xTg-AD mice. Treatment with P021 decreases the mortality rate without apparent side effects such as weight loss or decrease in food consumption in 3xTg-AD mice.

4.2 Results
4.2.1 Treatment with P021 can rescue reference memory deficit

As described in Chapter 3, I found that the 3xTg-AD mice were cognitively impaired as determined by the Morris Water Maze task very early in the disease process at ~3 months of age. Since the Morris Water Maze task measures spatial reference memory that is purely hippocampal dependent and spatial reference memory is affected early in AD patients, I sought to study the effect of P021 treatment on preventing or reversing this impairment. The Morris water Maze task was conducted after 9 months of treatment with P021, when the animals were aged 12 months.
During the task animals went through four training trials per day for four days for a total of 16 trials. There was no difference in the swim speed during the four training days (Fig. 23a). Analysis of the latency to escape showed that the learning curve of the 3xTg-AD-vh was slower compared to WT (Fig. 23b, p < 0.001) and 3xTg-AD-P021 mice (Fig. 23b, z = 4.78 p = 0.031). All three groups of mice learned the task, however. The analysis of distance by day showed also that the learning curve of 3xTg-AD-vh mice was slower than WT (Fig. 23c, p <0.001) and 3xTg-AD-P021 animals (Fig. 23c, p = 0.039) and they traveled farther to find the escape platform. These data showed that the 3xTg-AD-vh mice were impaired and that P021 was able to reverse this impairment.

To evaluate retention memory in the 3xTg-AD mice three probe trials were conducted. The first one (PT1) was twenty-four hours after the last training trial. The second probe trial (PT2) and the third probe trial (PT3) were separated by twenty days from each other and from the first probe trial. The percent of time spent in the target quadrant and the number of crossings of the previous place of the platform were determined to evaluate the performance of the animals. During PT1, the 3xTg-AD-vh spent significantly less time in the target quadrant compared to WT (Fig. 23d, post-hoc Bonferroni-adjusted test, p<0.05) and P021 treatment was able to reverse this impairment (Fig. 23d, p<0.01). A similar trend was seen in the number of visits to the previous place of the platform but there was no significant difference between the three groups (Fig. 23g). During PT2, the analysis of the percent of time in target quadrant showed that the 3xTg-AD-vh spent less time compared to the WT (Fig. 23e, p<0.05). Furthermore, the analysis of the number of crossings of the previous place of the platform showed that the 3xTg-AD-vh had made fewer visits compared to the WT and that P021 reversed this impairment (Fig. 23h, p = 0.094). For PT3, the percent of time in target quadrant and the
number of platform crossings showed a similar trend to PT1 and PT2 but were not significant (Fig. 23f, i). These results suggest that memory retention was compromised in 3xTg-AD-vh mice and that the 9 month treatment with P021 rescued this impairment.

**Fig. 23**: At 12 months of age after 9 months of treatment the 3xTg-AD-vh mice were impaired in the Morris Water Maze task and P021 prevented this impairment.

a) Swim speed, b) Latency to escape, c) Distance traveled, d) percent of time in target quadrant in PT1, e) percent of time in target quadrant in PT2, f) percent of time in target quadrant in PT3, g) number of platform crossings PT1, h) number of platform crossings PT2, and i) number of platform crossings PT3. Tq: target quadrant, AL: adjacent left, AR: adjacent right, Oq: opposite quadrant. *p<0.05; **p<0.01, ***p<0.001. (WT, n = 20; Tg-AD-vh, n=15; Tg-AD-P021, n = 14). Data are shown as mean ± SD.
4.2.2 Treatment with P021 can rescue short-term reference memory impairment

The neophobia test is used to measure the rodent’s fear of discovering novelty, in this case a novel object. This test was conducted during the first habituation session before the object location test. The statistical analysis of the percent of time spent in the center exploring the novel object showed that both the WT and 3xTg-AD-vh mice spent similar time exploring the new object, while the P021-treated spent significantly more time exploring the novel object compared to the two previous groups (Fig. 24a, p = 0.0086). The novel object location in the open field was conducted twenty four hours after the last habituation session. The Object location test comprised a sample phase and a test phase. The analysis of the data from the sample phase showed that, as expected, there was no difference in exploring the two identical locations between the three groups of mice (Fig. 24b). However, the data from the test phase showed that 3xTg-AD-vh mice spent more time investigating the familiar location than the novel one, while the WT and 3xTg-AD-P021 groups spent more time exploring the novel location than the familiar one (Fig. 24c, p = 0.0072).

Together these data showed that the 3xTg-AD-vh at the age of 18 months had impairment in short-term spatial memory and that the ~15 months treatment with P021 reversed this impairment.
Fig. 24. At 18-19 months of age after 15-16 months of treatment, when compared to WT, the 3xTg-AD-vh showed impairment in short-term spatial reference memory in the object location task and P021 was able to prevent this impairment as well as decrease neophobia in 3xTg-AD mice. a) Neophobia test, b) sample phase, and c) test phase. *p<0.05; **p<0.01, ***p<0.001. (WT, n = 20; Tg-AD-vh, n=15; Tg-AD-P021, n = 14). Data are shown as mean ± SD.

4.2.3 P021 can rescue episodic memory impairment

The Novel object recognition task is based on a rodent’s preference to explore novelty. It is thought to depend solely on the entorhinal cortex, hippocampus and frontal cortex. This test is considered to test only some aspects of the episodic memory because the experimenter has no access to the temporal dimension of the episode. The only accessible components are the memory, the place and the context in which an object is encountered and thus the test is considered to be a test of short-term memory (Ennaceur, 2010). The sample phase was conducted twenty-four hours after the last habituation phase and the analysis of the data showed, as
expected, no difference between the three groups in exploring the two identical objects (Fig. 25a). The trial test was conducted 15 min after the sample phase. I found that the 3xTg-AD-vh mice were impaired and that the WT mice were also impaired because of the effect of aging. They either spent a similar amount of time near the familiar and the novel objects or spent more time near the familiar one. However, the 3xTg-AD-P021 spent more time exploring the novel object than the familiar one (Fig. 25b, p = 0.0234). These data suggest that at 19-20 months of age the 3xTg-AD-vh and WT mice were impaired and that the chronic treatment with P021 rescued this impairment.

Fig. 25: The object recognition task at 19-20 months of age (after 16-17 months of P021 treatment) showed that the WT and 3xTg-AD-vh were impaired in episodic-like memory and that P021 rescued this impairment in 3xTg-AD mice. a) Sample phase and b) test phase. *p<0.05; **p<0.01, ***p<0.001. (WT, n = 20; Tg-AD-vh, n=15; Tg-AD-P021, n = 14). Data are shown as mean ± SD.

4.2.4 Prevention of cognitive impairment across time

Data from all three cognitive tests were converted to cognitive scores. Cognitive scores from each test were calculated as: mean of each mouse - mean of all mice) / SD (all mice). The cognitive score represents the number of SD from the mean. Then cognitive scores from all tests were compared across time with the two-way ANOVA. The results showed that very clearly
across time there was a decrease in cognitive performance in 3xTg-AD mice compared to WT (p = 0.017). However, in 3xTg-AD-P021 there was a marked enhancement of cognitive performance (Fig. 26, p = 0.001) especially at 15-17 months post-treatment.

Fig. 26. Comparison of cognitive scores over time in all the behavioral tests. *p<0.05; **p<0.01, ***p<0.001. (WT, n = 20; Tg-AD-vh, n=15; Tg-AD-P021, n = 14). Data is shown as mean ± SEM.

4.2.5 P021 treatment can decrease mortality and has no effect on body weight or food consumption.

Analysis of survival rates at the end of the study showed that P021 treatment decreased the mortality rate in 3xTg-AD-P021 mice. Indeed, the survival rate of 3xTg-AD-vh at week 71 was significantly less (41%) than the survival rate of 3xTg-AD-P021 (87%) ($\chi^2_{[1, df]} = 9.437, p = 0.0021$) (Fig. 27a). The body weight analysis showed no difference among the three groups (Fig. 27b). Similarly there was no difference in the food consumption among the groups (Fig. 27c).
Together these data suggest that the delay of the onset of cognitive impairment in 3xTg-AD - P021 may have been able to decrease the mortality rate in the treated animals without causing any serious side effects such as decrease in body weight or food consumption.

Fig. 27: Survival curve analysis showed an increase by 87% in 3xTg-AD-P021 compared to only 41% in 3xTg-AD-vh mice survived by 71 weeks with no side effects of P021 on food intake or body weight. a) survival curve, b) body weight, and c) food intake. *p<0.05; **p<0.01, ***p<0.001. (WT, n = 20; Tg-AD-vh, n=15; Tg-AD-P021, n = 14). Data are shown as mean ± SD.
4.3 Discussion

The clinical diagnosis of AD relies mainly on the appearance of signs of cognitive impairment and memory loss, especially loss of short-term and declarative memories. The severe decline in mental abilities, or what is referred to as dementia, is the major burden in AD. Indeed, it compromises daily activities for the patient and makes him/her totally dependent on caregivers in the severe stages. Delaying the onset of dementia and cognitive deterioration would be of major importance in AD. Furthermore, preventing AD would result in the decrease in the mortality rate due to this disease, since it is the sixth leading cause of death in the USA. Thus, a drug that could delay or reverse cognitive deterioration would be ideal because it could prevent AD and decrease the death rate due to this disease.

The present study showed that, reminiscent of AD, the 3xTg-AD mice are impaired in spatial reference memory as tested by the Morris Water Maze task. I further show that the initiation of treatment with the neurogenic/neurotrophic compound P021 right after the start of the cognitive impairment, around the period of synaptic compensation, for 9 months is able to prevent cognitive impairment completely in the 12 month old 3xTg-AD mice. Our results also show that impairment in short-term spatial memory, as tested with the novel object location task, is completely prevented after 15 months of treatment with P021 when the 3xTg-AD mice are 18 months old. Using the novel object recognition task that measures episodic-like memory, I show complete prevention of cognitive impairment after treatment of ~3 month old mice with P021 for 16-17 months. Most importantly, the analysis of survival rates shows that P021 treatment is able to increase the survival rate from 41% in 3xTg-AD-vh to 87% in 3xTg-AD-P021. These beneficial effects of long-term treatment with P021 on cognition and survival rate in 3xTg-AD mice are seen in the absence of any apparent side effects such as decrease in the food
consumption or body weight.

All of these data collectively suggest that initiation of the treatment with P021 very early in the disease process can delay the onset of cognitive impairment and decrease the mortality rate without apparent side effects in the 3xTg-AD mice. Thus, P021 could potentially delay the onset of cognitive impairment in AD patients.

The present findings that the 3xTg-AD mice are impaired at 3 months of age (Chapter 3) was also reported previously in the literature (Davis et al., 2013). The three-month time point apparently overlaps with the onset of cognitive impairment in these mice, since it was previously reported that at 2 and 2.5 months the 3xTg-AD mice are cognitively normal and that they were not born with cognitive impairment (Billings et al., 2005, Gimenez-Llort et al., 2007). Our findings are consistent with the previous report that the 3xTg-AD mice are cognitively normal until 3 months of age and then cognitive deterioration happens in an age-dependent manner due to the transgenic products or their derivative (Billings et al., 2005). According to the findings of this study and previous findings, the 3xTg-AD mice remained impaired throughout their lifespan as assessed by the Morris Water Maze and other behavioral tasks (Gimenez-Llort et al., 2007, McKee et al., 2008, Filali et al., 2012). It is very difficult in AD patients to detect the onset of cognitive impairment because of the lack of a reliable biomarker. In contrast, mice can be assessed behaviorally by a battery of tests to detect the early signs of cognitive impairment and correlate it with molecular and cellular changes. This then emphasizes the usefulness of this study, first because I was able to detect the very early onset of cognitive impairment and follow cognitive deterioration across time in 3xTg-AD mice, and second because I was able to study the effect of early therapeutic intervention in preventing cognitive deterioration.
The results of this study show that the oral treatment of 3 month old 3xTg-AD mice for 9 months with P021 can prevent cognitive deficit, and this is consistent with our previous reports, where we started the treatment with P021 or Peptide 6 later during the disease process (Blanchard et al., 2010b, Kazim et al., 2014). We previously showed that P021 is a neurogenic/neurotrophic compound that increases synaptic plasticity, neurogenesis and BDNF expression and decreases tau and possibly Aβ pathologies (Blanchard et al., 2010a, Blanchard et al., 2010b, Li et al., 2010, Bolognin et al., 2012, Bolognin et al., 2014). Since the cognitive impairment reported in this study starts very early in the disease process with the onset of synaptic loss without apparent Aβ and tau pathologies, I speculate that the preventive effects of P021 on cognition at 12 months of age are mainly mediated by its beneficial effects on synaptic plasticity and neurogenesis. Similarly, it was reported previously that the neurogenic and neurotrophic effects of Amitriptyline (FDA approved tricyclic antidepressant drug) are able to reverse cognitive impairment in 3xTg-AD mice at 14 months of age (Chadwick et al., 2011). Furthermore, it is well documented that BDNF is an essential effector of spatial learning and that its level increases three and six days after training in the hippocampus and the entorhinal cortex (Kesslak et al., 1998, Harvey et al., 2008). The fact that P021 acts by increasing BDNF expression could also explain the rescue of the cognitive deterioration seen at 12 months of age in 3xTg-AD mice in the present study.

The present study shows that in the novel object location task at 18 months of age the 3xTg-AD mice are impaired. Furthermore, the 15 month treatment with P021 can rescue this impairment. The impairment in the novel object location task in the 3xTg-AD mice found in this study is consistent with previous findings in 15-18 month old 3xTg-AD mice (Gulinello et al., 2009). Since at this stage Aβ and tau pathologies are prominent as well as synaptic loss, I
speculate that the beneficial effects of P021 could be also because of its preventive effects on Aβ and tau pathologies; the effect of BDNF on prevention of cognitive impairment in 12-14 month old 3xTg-AD mice has been reported (Corona et al., 2010). Our previous studies showed that P021 acts by decreasing the hyperphosphorylation of tau through the GSK3β pathway (Kazim et al., 2014). P021 acts by increasing BDNF expression that acts through the PI3k-Akt pathway to increase the phosphorylation of GSK3β on serine 9 and inhibits its activity. Since GSK3β is a major tyrosine kinase that phosphorylates tau, that is most likely why we see the decrease in tau phosphorylation. The partial decrease in Aβ is also suggested to be through inhibiting GSK3β activity, since it has been shown previously that GSK3β mediates Aβ neuritic damage (DaRocha-Souto et al., 2012) and that its inhibition decreases both Aβ and tau pathologies and rescues cognitive impairment in other AD mouse models (Noble et al., 2005, Hu et al., 2009, Sereno et al., 2009, Onishi et al., 2011).

Since episodic memory is severely affected during the AD pathological process, I investigated the impairment in episodic-like memory in 3xTg-AD mice and the effect of 16-17 month treatment with P021 on alleviating it. This study showed that the 19-20 month 3xTg-AD-vh mice were impaired in episodic memory, but surprisingly I found that the WT mice were also impaired because of the effect of aging. The treatment with P021 rescued the episodic-like memory deficit seen at this age. Our findings are corroborated by previous findings that showed that at 12 months of age both WT and 3xTg-AD mice were impaired in an episodic-like memory task, the WWWWhich occasion (What-Where-Which occasion) task (Davis et al., 2013). Most importantly, episodic memory impairment has been reported previously in cognitive aging because it is sensitive to gray and white matter loss as well as to subtle changes in dendritic spines that affect synaptic plasticity (Burke and Barnes, 2006, Charlton et al., 2010, He et al.,
2012). The present finding that the WT mice were spared in short-term spatial memory and impaired in episodic like memory was also reported previously in rats (Winters et al., 2004). Indeed, depending on the place of the lesions, rats can be impaired in the novel object recognition but not the novel object location and vice versa. The hippocampus is believed to be essential for object location but not object recognition. However, the entorhinal cortex was shown to be important in object recognition but not object location, and this suggests a functional dissociation between the two regions (Winters et al., 2004).

To our knowledge this is the first study showing that P021 treatment for 18 months can dramatically increase the survival rate in 3xTg-AD mice by more than 40%. The 3xTg-AD-vh mice showed a low level of survival, especially from 12-18 months of age at the peak of Aβ and tau pathologies. Our findings are in agreement with previous literature that showed a decrease in the long term survival rate in AD patients during a 14 year period from an expected rate of 16.6% to 2.2%. Furthermore, the decrease in the survival rate becomes more dramatic in AD with multi-infarct dementia: 1.7% versus an expected 13.3% (Molsa et al., 1986). The increase in disease severity was found to be associated with an increase in the level of mortality similar to what I reported here, i.e., that the highest mortality level was registered at the peak of Aβ and tau pathologies. The increase in the long-term survival rate seen after P021 treatment further emphasizes that a therapeutic drug that would be able to delay the onset of cognitive impairment and underlying pathologies in AD could decrease the level of death due to this disease. The lack of a therapeutic drug makes AD the sixth major leading cause of death in the USA and the most common cause in people aged 65 years or older.

Since P021 is derived from the CNTF, and CNTF in clinical trials showed serious side effects such as muscle pain, anorexia, loss of appetite and weight loss (see Chapter 1), I
measured body weight and food consumption throughout the study. The results revealed no
effect of P021 on food intake or body weight; this further emphasizes that P021 is a potentially
safe drug that can be developed to treat AD.

4.4 Conclusion
In conclusion, our data show that early treatment with P021 right after the onset of
cognitive impairment during the period of synaptic compensation can prevent cognitive
impairment for the different stages of the disease, mild (12 months) and moderate to severe (18-
20 months), in 3xTg-AD mice. I also found that P021 treatment for the 18 month period can
dramatically decrease the mortality level by more than 40% in 3xTg-AD mice. The beneficial
effects of oral treatment with P021 can occur in the absence of severe side effects such as loss of
appetite or weight loss. These findings show that P021 is a promising therapeutic compound for
the treatment of AD and related neurodegenerative conditions.
Chapter 5. Effect of P021 on Aβ and tau pathologies

5.1 Introduction
AD is caused by severe neuronal loss in the limbic and neocortical areas but the real cause of neurodegeneration is still unknown. AD accounts for 60-80% of dementia cases; multi-infarct dementia or vascular dementia comes in second place, accounting for 20-30% of dementia cases (www.alz.org; Ott et al., 1995). Multi-infarct dementia is caused when the brain tissue loss reaches 100-150 ml. The risk of multi-infarct dementia usually increases with the number of infarctions that occur over time. Small infarcts usually occur because of the decrease in blood flow to the brain. Decrease in the blood flow to the brain leads to a decrease in the brain’s oxygen level, which leads to brain tissue loss due to ischemia. The decrease in the blood flow, it is suggested, results from the increase in Aβ deposition in the brain’s blood vessels, called cerebral vascular angiopathy. According to the Aβ hypothesis, the excess production of Aβ or its soluble forms is the trigger for the neurofibrillary degeneration and dementia associated with AD.

Aβ is a metabolic product of the cleavage of the amyloid precursor protein (APP). The APP transcript is alternatively spliced into three different mRNAs: APP770 and APP751, which each contain a Kunitz-type serine protease inhibitor domain (APP-KPI), and APP695, which lacks this domain. APP 695 is expressed primarily in neurons, whereas APP 770 and 751 are expressed in neurons and glia. In AD the mRNA levels of the three APPs have been shown to be increased, but in neurons there was a shift from the APP 695 mRNA into APP-KPI mRNAs, APP770 and APP751 (Matsui et al., 2007). There are two pathways for APP processing, either amyloidogenic or non-amyloidogenic (Fig. 28). In the amyloidogenic pathway APP is cleaved by
β-secretase to produce the C99 fragment that is later processed by the γ-secretase to produce Aβ peptides, including Aβ 40 and 42, the most abundant fragments in Aβ plaques (Robakis, 2010). The non-amyloidogenic pathway involves cleavage with α-secretase within the Aβ-sequence on the APP, followed by γ-secretase, to produce the APP C-terminal fragment, thus inhibiting β-secretase and the formation of Aβ peptides.

Fig. 28. Amyloidogenic and non-amyloidogenic pathways (Zhang, 2012).

Currently the Aβ hypothesis, which postulates amyloid deposition and soluble Aβ as causing disease, represents the basis of several therapeutic strategies, especially several prevention studies such as DIAN and API (see Chapter 1). However, this hypothesis remains controversial because the Aβ load fails to correlate with the severity of dementia or neuronal and synaptic loss (Terry et al., 1981, Braak and Braak, 1991, Neve and Robakis, 1998). Furthermore, around 30% of normal aged people express as much amyloid deposition as in a typical AD brain.
(Crystal et al., 1988, Arriagada et al., 1992a, Davis et al., 1999). Behavioral abnormalities in Tg mice were also shown to be independent of Aβ deposition (Robakis, 2010). Several transgenic mice that express high levels of Aβ deposition failed to show significant neurodegeneration. Aβ has been shown to exacerbate tau pathology in Tg mice. However, these Tg mouse models were generated overexpressing mutated tau, while to date no tau mutations have been found in AD (Iqbal and Grundke-Iqbal, 2010). Furthermore, in the familial cases of FTDP-17 caused by tau mutations in chromosome 17 there is only tau pathology and no β-amyloidosis. In other dementia cases such as hereditary cerebral hemorrhage with amyloidosis of Dutch origin (HCHWA-D) and sporadic cerebral amyloid angiopathy (SCAA) β-amyloidosis is extensive in the absence of any tau pathology (Coria et al., 1987, van Duinen et al., 1987). Moreover, a recent study reported that there is no colocalization between Aβ deposition and tau NFT in most AD brain regions, suggesting that Aβ is a poor variable to explain the development of tau pathology (Lace et al., 2009). The successive failures in anti-Aβ therapeutic approaches in phase II and phase III clinical trials makes it unlikely that Aβ is the major trigger of AD dementia and neurofibrillary degeneration, especially as the anti-Aβ therapies were successful in clearing Aβ from the parenchyma of the brains of AD patients, as shown with postmortem studies, and yet no cognitive improvement or halting of the disease progression was observed (Holmes et al., 2008).

A main support of the Aβ hypothesis is the presence of presenilin (PS) mutations in the FAD cases, which is believed to cause the increase in the generation of the neurotoxic Aβ fragment 1-42 and increase the ratio of Aβ 42/40. PS1 is a transmembrane protein involved in the γ secretase processing of APP and the production of Aβ peptide. In FAD cases more than 130 PS1 mutations were reported. To explain the mechanism of neurodegeneration in FAD cases the Aβ hypotheses suggests that the PS1 mutations favor the cleavage of APP by γ-secretase. This
leads to an increase in the generation of the neurotoxic Aβ fragment that promotes cerebral amyloidosis and formation of Aβ oligomers (Hardy and Selkoe, 2002, Koo and Kopan, 2004). However, a recent study reported that only three out of eight PS1 mutations showed an increase in Aβ production. This shows that mutation in the PS is not always accompanied by an increase in Aβ 42 generation or even increases the ratio of Aβ 42/40. Some PS mutations even cause a loss instead of a gain of function in γ-secretase activity (Shioi et al., 2007). Indeed, it has been reported that PS2 mutations are not accompanied by an increase in Aβ42 production or an increase in Aβ42/Aβ40 ratios (Walker et al., 2005). The increase in Aβ production also does not correlate with the age of the onset of disease in AD cases with PS1 mutations (Citron et al., 1997, Mehta et al., 1998). Furthermore, even though the APP_{Swe} mutation increases the production of Aβ40 and Aβ 42 it does not result in a change in the Aβ42/Aβ40 ratio (Kumar-Singh et al., 2006). Thus it was suggested that the FAD mechanism of neurodegeneration may be independent of the effect of presenilins on Aβ production (Shioi et al., 2007).

Aβ peptides are expressed by all cells. They are normal components of human serum and CSF, there is little evidence of disease associated abnormalities of Aβ and no toxic oligomers are specific to AD. It is unclear under what conditions these peptides become toxic. Several studies currently suggest that Aβ deposition could be secondary to neuronal dysfunction of AD but not a primary agent that causes the disease (Robakis, 2010). The FAD mutations in PS1, PS2, and APP may not cause neurodegeneration because of their effect on increasing the generation of Aβ but by altering the signal transduction pathways, especially those involving protein phosphatase-2A (PP2A) and glycogen synthase kinase-3β (GSK-3β) (Iqbal et al., 2014b). In a recent study another mechanism of cell death was shown to be promoted by APP. Indeed, one fragment of
APP was shown to interact with the cell death receptor 6 to promote neurodegeneration in mice (Nikolaev et al., 2009).

Hyperphosphorylation of tau, on the other hand, is a major cause of neurodegeneration in tauopathies (Table 6) (Lee et al., 2001, Brandt et al., 2005, Goedert and Jakes, 2005, Brunden et al., 2010). NFT are one of the earliest pathological substrates of aging and a neuropathological continuum from aging to MCI and AD (Arriagada et al., 1992b, Guillozet et al., 2003). In the rare form of AD with plaques only and hyperphosphorylated tau without tangles (POAD) cognitive deterioration is slower and the onset of the disease tends to start later than typical AD patients with NFT and amyloid plaques (PTAD). Patients in the POAD group tend to die at older ages and there is a trend toward shorter duration of the disease (Tiraboschi et al., 2004). Furthermore, the severity of cognitive impairment is directly correlated with the number of NFT (Arriagada et al., 1992a). In contrast, Aβ plaque deposition does not correlate either with neuronal loss and NFT or with the severity of the disease (Gomez-Isla et al., 1997).

Tau is a major microtubule-associated protein in the brain. Tau’s normal function is to interact with microtubule binding domains to stabilize and maintain their structure. In the normal brain tau contains 2-3 moles of phosphate per mole of the protein; in the AD brain it is 3-4-fold hyperphosphorylated. The hyperphosphorylation of tau leads to its binding and sequestration of normal tau and the other two microtubule associated proteins (MAP1 and MAP2), which causes disruption of microtubules (Kopke et al., 1993, Alonso et al., 1994, Alonso et al., 1996, Alonso et al., 1997). Hyperphosphorylation of tau is suggested to happen because of an imbalance between tau protein kinase and phosphatase activities. In AD two main features are observed in tau pathology: either there is microtubule destabilization, which is considered to be a loss of function, or tau oligomerization, which is considered to be a gain of toxic function (Avila, 2000).
Tau is synthesized in the human brain as six isoforms generated by alternative splicing of its pre-mRNA at exons 2, 3, and 10, generating the protein with 352 to 441 amino acids in length. Variation in the splicing of exons 2/3 inclusion/exclusion are called 0N (no inclusion of exons 2 and 3), 1N (inclusion of exon 3), and 2N (inclusion of exons 2 and 3). The alternative splicing of exon 10 generates tau isoforms either with three (3-R) or four (4-R) carboxyl-terminal MT-binding repeat motifs. The six isoforms are 4R2N, 4R1N, 4R0N, 3R2N, 3R1N, and 3R0N (Iqbal et al., 2005, Brunden et al., 2010, Wolfe, 2012). In human tau expression is under developmental control: in the fetus tau is exclusively 3R0N but in adults all six isoforms are expressed.

Neither Aβ nor NFT are specific to AD. They are also found in normal individuals in small numbers. Previous reports emphasized that a better correlate of cognitive impairment in AD is synaptic and neuronal loss (DeKosky and Scheff, 1990, Terry et al., 1991, Gomez-Isla et al., 1997). Thus, a combination of a drug that could inhibit Aβ and tau pathologies and neurodegeneration and increase the regenerative capacities of the brain would be ideal to treat AD and related neurodegenerative diseases.

We previously reported that P021 has a neurogenic/neurotrophic effect and it can decrease Aβ and tau pathologies in 3xTg-AD mice in the severe stages of the disease. In the present study I investigated the effect of P021 on preventing tau phosphorylation and Aβ plaque formation by starting the treatment before the onset of these two pathologies in 3xTg-AD mice.
<table>
<thead>
<tr>
<th>Tauopathies</th>
<th>Cause</th>
<th>Areas of the Brain Mainly Affected</th>
<th>Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontotemporal dementia with Parkinsonism linked to chromosome-17 tau (FTDP-17)</td>
<td>Missense mutation in tau that makes it a more favorable substrate for hyperphosphorylation by protein kinases, characterized by abnormal accumulation of 3R tau</td>
<td>Frontal and temporal lobe</td>
<td>Irrational decisions and socially inappropriate behaviors</td>
</tr>
<tr>
<td>Pick’s disease</td>
<td>Missense mutation of tau (9 are known) Presence of tau filaments Presence of tau-immunoreactive Pick bodies</td>
<td>Frontotemporal lobar and limbic atrophy</td>
<td>Dementia and loss of language</td>
</tr>
<tr>
<td>Cortico-basal degeneration</td>
<td>Increase in the expression of hyperphosphorylated tau in astrocytes and glia cells Overexpression of the 4R repeat tau that aggregates into filaments Extensive accumulation of tau-immunoreactive neuropil threads throughout gray and white matter</td>
<td>Cerebral cortex, deep cerebellar nuclei, and substantia nigra, in association with prominent neuronal achromasia</td>
<td>Clinical signs very similar to PSP. Marked disorder in movement and cognitive dysfunction</td>
</tr>
<tr>
<td>Progressive Supranuclear Palsy (PSP)</td>
<td>Caused in part by a variant in the tau gene called H1 haplotype Overexpression of the 4R repeat tau that aggregates into filaments mainly neuropil threads and NFT</td>
<td>Atrophy of basal ganglia, Subthalamus and brain stem, with corresponding neuronal loss and gliosis</td>
<td>Supranuclear gaze palsy and prominent postural instability</td>
</tr>
<tr>
<td>Dementia Pugilistica/traumatic brain injury/chronic traumatic encephalopathy</td>
<td>Tau pathology mainly as NFT</td>
<td>Multiple concussions or other head injuries to the brain</td>
<td>Neurodegeneration with dementia and Parkinson’s disease-like symptoms</td>
</tr>
</tbody>
</table>

Table 6. Major tauopathies where tau pathology occurs in the absence of Aβ pathology.
5.2 Results

5.2.1 Prevention of Aβ and tau pathologies by P021 in 3xTg-AD mice

Since Aβ and tau pathologies are the two hallmarks of AD I investigated the effect of P021 on the prevention of these two pathologies. The effect of P021 was studied by the treatment of 3 month old 3xTg-AD mice for 9 months. Aβ and tau pathologies in the 3xTg-AD mouse are localized in the CA1 and the subiculum regions as reported previously (Oddo et al., 2003a). The immunohistochemical staining with 4G8 antibody was used to detect Aβ pathology but it also recognizes APP. Immunohistochemical staining with AT8 antibody was used to detect tau hyperphosphorylation in the Ser202/Thr 205 site.

I found that at 12 months of age in the CA1 region only APP immunostaining could be seen and the analysis of the mean pixel intensity showed that P021 had no significant effect (Fig. 29a). In the subiculum region the staining, however, was both for APP and Aβ plaques. During the analysis the gain was lowered for each picture so that only the Aβ plaque load appeared. Then Aβ load was quantified and analyzed as percent of area occupied. I found that at 9 months after treatment the P021 was able to completely prevent Aβ pathology in the subiculum region (Fig. 29a, t-test, p = 0.0037).

The AT8 staining in the CA1 region was quantified as percent of area occupied. The results showed a trend toward a significant effect of P021 on preventing tau pathology in this region (Fig. 29b, t-test, p = 0.05). In the subiculum, however, the data were analyzed as mean pixel intensity and there was no apparent effect of P021 treatment at this time point (Fig. 29b).

The PHF1 staining in the CA1 region and the subiculum was quantified as percent of area occupied. The results showed a significant effect of P021 on preventing tau hyperphosphorylation in both regions (Fig. 29c, t-test, p = 0.03 and p < 0.0001, respectively).
Fig. 29. Effect of P021 on the prevention of Aβ and tau pathologies at 12 months.

a) 4G8 staining CA1 (upper panel) and subiculum (lower panel). b) AT8 staining in the CA1 (upper panel), and subiculum (lower panel). c) PHF1 staining in the CA1 (upper panel) and the subiculum (lower panel). *p < 0.05; **p < 0.01; ***p < 0.001. Scale bar = 100μm. (Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Data are shown as mean ± SD.
5.2.2 Preventive effects of P021 on Aβ and tau pathologies after 15 months of treatment (at 18 months of age)

The analysis of the percent of area occupied by the Aβ load in the CA1 region (Fig. 30a) showed no difference between the 3xTg-AD-vh and Tg-AD-P021. However, there was a strong preventive effect of P021 in the subiculum region seen as a decrease in the percent of area occupied by the Aβ load (Fig. 30a, t-test, p = 0.0028).

The analysis of the effect of P021 treatment after 15 months on tau hyperphosphorylation using the AT8 staining showed that there was a strong significant effect both in CA1 (Fig. 30b, t-test, p = 0.0015) and subiculum (Fig. 30b, t-test, p = 0.0024) regions. Similarly the analysis of the percent of area occupied by PHF1 positive immunohistochemical staining showed that P021 was able to prevent tau pathology in the CA1 region (Fig. 30c, t-test, p = 0.0018) and the subiculum (Fig. 30c, t-test, p = 0.0001).
Fig. 30: Effect of P021 on the prevention of Aβ and tau pathologies at 15 months post-treatment.

a) 4G8 staining CA1 (upper panel) and subiculum (lower panel). b) AT8 staining in the CA1 (upper panel), and subiculum (lower panel). c) PHF1 staining in the CA1 (upper panel), and subiculum (lower panel). *p < 0.05; **p < 0.01; ***p < 0.001. Scale bar = 100 µm. (Tg-AD-vh, n = 4; Tg-AD-P021, n = 5). Data are shown as mean ± SD.
5.2.3 Prevention of Aβ and tau pathologies after 18 months of treatment with P021 (21 months of age)

At 18 months after treatment, P021 was able to prevent Aβ pathology in the CA1 (Fig. 31a, t-test, p = 0.0018) and in subiculum (Fig. 31a, t-test, p < 0.0001) in the 3xTg-AD.

When analyzing the percent of area occupied for tau hyperphosphorylation using AT8 staining I found that P021 was able to prevent tau pathology in the CA1 (Fig. 31b, t-test, p = 0.0002) and the subiculum (Fig. 31b, t-test, p = 0.0002). The analysis of the PHF1 positive staining as percent of area occupied showed a significant effect of P021 on preventing tau pathology in the CA1 (Fig. 31c, t-test, p < 0.0001) and the subiculum (Fig. 31c, t-test, p < 0.0001). Western blots of the hippocampus showed similar findings (Fig. 31d, p = 0.0003).
Fig. 31: Effect of P021 on the prevention of Aβ and tau pathologies at 18 months post-treatment.
a) 4G8 staining CA1 (upper panel) and subiculum (lower panel). b) AT8 staining in the CA1 (upper panel), and subiculum (lower panel). c) PHF1 staining in the CA1 (upper panel) and subiculum (lower panel) and d) Western blots of the hippocampus developed with PHF1 and 134d and quantification. *p < 0.05; **p < 0.01; ***p < 0.001. Scale bar = 100μm. (WT, n = 5; Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Data are shown as mean ± SD.
5.2.4 Effect of P021 treatment across time on Aβ and tau pathologies

Regression analysis of the Aβ pathology across time in the subiculum showed that the Aβ load increased with age in the 3xTg-AD-vh (Fig. 32a; t = 4.61, p < 0.0001, AdjR² = 0.42) and that P021 treatment was able to decrease it across time starting from 12 months until 21 months (Fig. 32a; t = -4.32, p < 0.0001, AdjR² = 0.38).

The analysis of AT8 as a percent of area occupied across time in the CA1 area with the regression analysis showed that the AT8 load increased over time from 12-21 months of age in the 3xTg-AD-vh (Fig. 32b; t=5.48, p <0.0001, AdjR² = 0.42). However, P021 treatment was able to lower the AT8 load across time (Fig. 32b, t= -3.24, p = 0.003, AdjR² = 0.26). Furthermore, I found that the AT8 load increased at a slower rate in the 3xTg-AD-P021 compared to the 3xTg-AD-vh (Fig. 32b, t = -2.37, p = 0.027, AdjR² = 0.86).

The analysis of the PHF1 load across time as percent of area occupied in the CA1 area showed that it increased over time in the 3xTg-AD-vh (Fig. 32c, t = 6.64, p < 0.0001, AdjR² =0.61) and that P021 treatment was able to inhibit it (Fig. 32c, t = -2.51, p < 0.02, AdjR² = 0.16). Furthermore, P021 treatment was able to slow down the rise of the PHF1 curve across time (Fig. 32c, t = -4.23, p < 0.0001, AdjR² = 0.92). In the subiculum area the PHF1 load was higher in the 3xTg-AD-vh versus 3xTg-AD-P021 across time (Fig. 32d, t = -12.59, p < 0.0001, AdjR² = 0.85). Furthermore, P021 treatment was able slow down the rise of the PHF1 curve across time (Fig. 32d, t = -2.24, p = 0.034, AdjR² = 0.93).
Fig. 32: Analysis of the preventive effects of P021 across time. a) 4G8 staining in the subiculum in 3xTg-AD-vh (upper panel) and in Tg-AD-P021 (lower panel). b, c, d) AT8 (b) and PHF1 (c, d) staining in CA1 (b, c) and subiculum (d) in 3xTg-AD-vh (upper panel) and in Tg-AD-P021 (lower panel) *p < 0.05; **p < 0.01; ***p < 0.001. Scale bar = 100μm. (Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Quantification are shown as mean ± SD.
5.3 Discussion

Several drug driven therapeutics in AD have aimed to clear either Aβ plaques or NFT from the brain in a hope to stop the progression of AD pathologies. Since all the attempts to develop therapeutics based solely on removing Aβ from the brain have failed, and since AD is known to be a multifactorial disease, finding a drug that could affect both pathologies as well as increasing neurogenesis and neuronal plasticity could be promising. We showed previously that P021 increased neurogenesis and neuronal plasticity and rescued cognitive impairment in several AD models (Blanchard et al., 2010b, Bolognin et al., 2012, Kazim et al., 2014), a Down Syndrome mouse model (Blanchard et al., 2011) and a cognitive aging animal model (Bolognin et al., 2014). Here I show that P021, when administered early during the period of synaptic compensation before any overt Aβ or tau pathologies, can prevent them at 9, 15, and 18 months post-treatment and also limit the progression of the pathologies across time in 3xTg-AD mice. To our knowledge this is the first report of a neurotrophic/neurogenic compound that is able to prevent both Aβ and tau pathologies and slows their progression with time.

The appearance of senile plaques in the brain along with NFT is one of the diagnostic features of AD. I investigated the progression of Aβ pathology at three different time points — 12, 18, and 21 months — and the preventive effect of P021 on it in 3xTg-AD mice. At 12 months of age I showed a prevention of Aβ pathology in the subiculum but not in the CA1. The pathology in the CA1 region was not robust; I saw only APP staining. This may explain the effect of P021 on the subiculum and not on CA1. At 18 months and 21 months, however, the pathology was more robust and almost the whole CA1 and subiculum areas were involved. I found that P021 has a strong effect in preventing Aβ pathology. This finding is not in agreement with what we reported previously using this peptide. Indeed, when we started the treatment of
3xTg-AD mice after the disease onset (6-7 months of age) for 6 weeks, we found no effect of P021 on Aβ (Blanchard et al., 2010b). Treatment of 3xTg-AD mice starting at 9-10 months of age for 12 months showed that P021 was able to reduce Aβ generation (Kazim et al., 2014). The findings of the present study emphasize the importance of early treatment for prevention of Aβ pathology. The curve of the progression of Aβ pathology across time showed that the Aβ load increased across time in 3xTg-AD-vh. However, in P021 treated animals the progression of the disease was slowed down, especially from 18-21 months of age in the subiculum region.

Since tau hyperphosphorylation correlates with synaptic loss in animal models (Kimura et al., 2010) and the density of NFT correlates with the severity of dementia in AD patients (Arriagada et al., 1992a, Bierer et al., 1995, Dickson et al., 1995, Giannakopoulos et al., 2003, Guillozet et al., 2003) I studied the effect of chronic P021 treatment on tau phosphorylation with the phospho-specific tau antibodies AT8 and PHF1. P021’s potential preventive effects were also studied across three different time points: 9, 15, and 18 months post-treatment.

I found that at 12 months of age in the 3xTg-AD the NFT formation starts in the subiculum and only a very small area of the CA1 region is occupied. When I analyzed the immunohistochemical staining with AT8, I found a high variation from animal to animal but after 9 months of treatment 3xTg-AD-P021 showed a trend toward an effect in the CA1 but none in the subiculum; however, using the PHF1 immunohistochemical staining, I found that P021 was able to prevent tau pathology in the CA1 and the subiculum.

At 18 months of age in 3xTg-AD mice the pathology is already prominent in the subiculum and covers a large area in the CA1 region. I found that 15 months of continuous treatment with P021 significantly prevented tau pathology. These findings are in agreement with
a previous study in which we showed that at 15-16 months of age P021 treatment for six months was able to reverse tau pathology in 3xTg-AD mice (Kazim et al., 2014).

At 21 months of age treatment with P021 for 18 months prevented tau pathology both in the CA1 region and the subiculum. At this stage AT8 load was evident in the two regions in 3xTg-AD-vh mice. These results are consistent with our previous findings (Kazim et al., 2014).

The most important finding of the present study, though, is that P021 was able to slow the progression of the disease across time. This finding is of major importance because this shows that P021 could be a potential drug for AD that could stop or halt the progression of the disease.

I speculate that the preventive effects of P021 on Aβ and tau pathologies are through the inhibition of GSK3β, as we reported previously (Kazim et al., 2014). P021 acts by increasing the expression of BDNF, which leads to the increase in the phosphorylation of GSK3β on serine 9, which results on its inhibition. Since GsK3β is a major tau protein kinase for phosphorylation of the proline-directed sites, its inhibition results in a decrease in tau phosphorylation (Munoz-Montano et al., 1997). GSK3β also is implicated in Aβ mediated neuritic damage (DaRocha-Souto et al., 2012), APP processing and Aβ deposition (Phiel et al., 2003). Its inhibition is shown to reduce both Aβ and tau pathologies, neuronal survival and cognitive impairment (Sereno et al., 2009).

5.3 Conclusion

Collectively our data show that chronic early oral treatment with P021 results in prevention of Aβ and tau pathologies. I also show, by the analysis of its effect across time, that P021 is able to slow down the progression of the disease in the 3xTg-AD mouse model. This presents P021 as a potential disease modifying drug for AD.
Chapter 6. Effect of early treatment with P021 on neuroregeneration and neurodegeneration

6.1 Introduction

Increasing evidence suggests that the beginning of the pathological cascade of AD starts many years before the appearance of the clinical symptoms of cognitive impairment. The very early stages of AD are thought to start with synaptic dysfunction, followed by neuronal loss, Aβ and tau pathologies and cognitive impairment (Selkoe, 2002). Although the severity of dementia has been shown to correlate with the density of NFT or NFT plus senile plaques, neither of the two pathologies is specific to AD. They are also reported in normal aged individuals. For instance, in the oldest old people dementia is associated with very little pathology, while the absence of dementia was reported in the presence of high levels of Aβ and tau pathologies (Green et al., 2000, Silver et al., 2002). Furthermore, it has been shown that there is a significant overlap in the BRAAK stages between demented and non-demented patients (Gold et al., 2000, Riley et al., 2002). Synaptic loss, on the other hand, has been shown to be a consistent feature that differentiates demented and non-demented people and significantly correlates with the severity of dementia (DeKosky and Scheff, 1990, Terry et al., 1991, Scheff and Price, 2003). Synaptic loss correlates well with other types of dementia and age-associated decrease in cognitive performance, called normal cognitive aging (Coleman et al., 2004).

Postmortem analysis has revealed a drastic decrease in presynaptic and postsynaptic markers in very early AD stages, suggesting the degeneration of the whole synaptic element (Davidsson and Blennow, 1998). A decrease in the number of synapses of ~ 25-30% was reported in temporal and frontal cortical biopsies and around 15-35% loss in the number of synapses per neuron within 2-4 years of the onset of AD (Davies et al., 1987). Synaptic loss is
even more pronounced in the hippocampus, reaching 44% to 55% (Scheff et al., 2007). Synaptic degeneration is thought to start in the entorhinal cortex, with the dendrites as the first to undergo degeneration, since they make up to 90% of synaptic contact. Dendritic loss is found to occur in parallel with synaptic loss, and major synaptic loss occurs very early in AD (Davies et al., 1987, Masliah et al., 2001). It has been previously shown that the ratio of synapses to neurons decreases by 48%, suggesting that synaptic loss is not happening only in the degenerated neurons but also in the remaining neurons (Coleman and Yao, 2003). Thus, in AD neurons may survive for a long period of time with a progressive deterioration of structure and function (Coleman et al., 2004). Surviving neurons account for as much as 38% of synaptic loss in AD. Cognitive deficit is thus suggested to occur not only because of synaptic loss but because of the impaired capacity of the still surviving synapses.

In spite of the reduced capacity of synaptic plasticity there is still a residual plastic capacity, as shown by the synaptic compensation phenomenon that happens in the early AD stages and in transgenic mice (see Chapter 3). In a previous study, although synaptophysin was reported to decrease in the frontal cortex of demented people, an increase was found in cognitively impaired non-demented 90+ year old individuals. This suggests that synaptic compensation counteracts synaptic loss and accounts for preserved cognition in these individuals in the presence of extensive pathology (Head et al., 2009). In the Rush Religious Orders Study an increase in the level of synaptophysin in the superior frontal cortex of MCI patients was reported compared to normal individuals (Counts et al., 2006). Furthermore, an increase in the level of synaptophysin in elderly people with extensive pathology and normal cognition was found compared to patients diagnosed with clinical and pathological AD (Lue et al., 1996, Lue et al., 1999). However, the increase in synaptophysin expression does not necessarily mean an
increase in the number of synapses, since an increase in the synaptic size to keep an intact total synaptic contact area in the very early stages of the disease has been found (Scheff and Price, 2003).

Brain mass loss/year is estimated to be ~ 0.5% in normal aged individuals and ~5-fold higher in AD (Fox et al., 1996). During the period of 7-10 years of disease progression, brain loss is estimated to be 200–400 g (Chan et al., 2001). The hippocampus is the major brain region that undergoes neuronal loss in AD and the atrophy is as drastic as 10% per year. More than 50% of the neurons in the brain are lost and the loss increases with the severity of the disease. Neuronal loss in the cortical association areas is thus directly correlated with memory loss (Gomez-Isla et al., 1997). It was shown previously that neuronal loss is prominent in layer II of the entorhinal cortex in mild AD, which distinguishes it from that layer in non-demented aged individuals. Furthermore, the number of neurons is inversely correlated with the number of NFT but not with senile plaques (Gomez-Isla et al., 1996). Neuronal loss has been linked to the abnormal hyperphosphorylation of tau. As stated previously (chapter 5), hyperphosphorylated tau sequesters normal tau, which causes the breakdown of microtubules. This would compromise axonal transport and lead to retrograde degeneration and synaptic loss (Iqbal and Grundke-Iqbal, 2002). These events are believed to lead to brain volume loss and dementia.

A key mechanism of deficient neurogenesis, neuronal loss and synaptic plasticity could be the imbalance of neurotrophic factors (Li et al., 2008). It has been shown that neurons maintain two pools of NGF: a constitutive pool in the soma that may maintain neuronal survival and a regulatable pool in the dendrites to maintain plasticity. A small change in the NGF level in transgenic mice is linked to impaired synaptic plasticity and cognitive performance. Furthermore, in AD the BDNF polymorphism from Valine to Methionine occurs with high
frequency and it has been shown to inhibit the cleavage of Pro-BDNF to BDNF (Coleman et al., 2004). An imbalance of neurotrophic factor is suggested to be the cause of the deficit in neurogenesis (Li et al., 2008).

I postulated that providing the diseased brain with neurotrophic activity could boost the process of neuroregeneration to counteract synaptic and neuronal loss and enhance neurogenesis. Since synaptic and neuronal loss and the compensation phenomenon happen very early during the disease process, I attempted to intervene at this early age in the 3xTg-AD mice (~3 months) with the neurogenic/neurotrophic peptide P021 to learn if I could prevent the disease progression to advanced stages.

The purpose of this study is thus to investigate the effect of P021 on prevention of synaptic and neuronal loss, neurogenesis deficit and neurodegeneration in 3xTg-AD mice by starting the treatment at 3 months of age and maintaining it for several months. Synaptic loss was studied immunohistochemically and biochemically using different synaptic markers such as synaptophysin, synapsin 1, GluR1, PSD-95, NR1 and MAP2 at 3 months of age just before treatment with P021, at 12 months (9 months after treatment), at 18 months (15 months after treatment), and at 21 months (18 months after P021 treatment). Neurodegeneration at the above time points was also studied using Fluorojade C staining. Neurogenesis was studied at 12 months of age using DCX and Ki-67 staining.

6.2 Results
6.2.1 Preventive effect of P021 on synaptic plasticity

_Synaptic loss in 3xTg-AD at 3 months of age before the start of the treatment_

Since my aim was to start the treatment with P021 very early in the disease process at around the period of synaptic compensation, I analyzed brain tissues of 3xTg-AD mice before
treatment. Based on my findings described in chapter 3, the onset of synaptic loss and its compensation starts at around three months in 3xTg-AD mice. I analyzed the level of expression of MAP2 immunohistochemically and found a trend toward a decrease in the CA1 (Fig. 33a, e, \( p = 0.05 \)) and a significant decrease in the DG (Fig. 33a, e, \( p = 0.0429 \)). Similarly, Western blot analysis of the hippocampus showed a significant decrease in the protein expression level of MAP2 (Fig. 34b, c, t-test, \( p = 0.01 \)). I found that the synaptophysin expression level decreased both in CA3 (Fig. 33b, e, \( p = 0.0209 \)) and DG (Fig. 33b, e, \( p = 0.0044 \)). The PSD-95 expression showed a significant difference between the WT and 3xTg-AD mice in the parietal cortex (Fig. 33c, e, \( p = 0.0395 \)). In the cortex the level of synaptophysin (Fig. 34a, c, \( p = 0.0014 \)) was reduced significantly while that of GluR1 showed a trend toward decrease in 3xTg-AD mice (Fig. 34a, c, \( p = 0.06 \)) as shown by Western blots. The level and the expression of synapsin 1 were also investigated immunohistochemically and biochemically but there was no significant difference between the 3xTg-AD and the WT. Furthermore, the level and the expression of NR1 in the parietal cortex decreased significantly in the 3xTg-AD mice compared to WT, as shown by immunohistochemical analysis (Fig. 33d, e, \( p = 0.0014 \)) and by Western blots of the cortex (Fig. 34a, c, \( p = 0.004 \)). The analysis of the level of the ratio of pCreb and Creb in the cortex by Western blots showed a significant decrease in the 3xTg-AD mice compared to WT (Fig. 34a, c, \( p = 0.002 \)). No significant difference was found in the level of PSD95 between the two groups by Western blots in the cortex and the hippocampus (Fig. 34a, c). Human-specific monoclonal antibody 43D to tau was used to check the 3xTg-AD mice genotype compared to WT by Western blots (Fig. 34a, b).
Fig. 33: Synaptic deficit at 3 months of age in 3xTg-AD mice.

a) Immunohistochemical analysis of the MAP2 level in the CA1 region (upper panel) and the DG (lower panel). b) Level of expression of synaptophysin in the CA3 region (upper panel) and the DG (lower panel). c) Level of expression of PSD-95 in the parietal cortex. d) Expression of NR1 in the parietal cortex and e) quantification of immunohistochemical staining. t-test. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100μm. (WT, n = 5; Tg-AD vh, n= 5). Quantification is shown as mean ± SD.
Fig. 34: Analysis of the protein level in 3 months old 3xTg-AD mice by Western blots. 

a) Cortex, Western blots 

b) Western blots analysis of the protein level in the hippocampus and c) quantification, *t-test, *p <0.05; **p <0.01; ***p <0.001. (WT, n = 6; Tg-AD vh, n= 6). Quantification is shown as mean ± SD.
Effect of P021 on synaptic plasticity after 9 months of treatment in 3xTg-AD

I investigated the effect of 9 months of treatment with P021 on synaptic plasticity immunohistochemically and biochemically. The immunohistochemical analysis of the expression of PSD-95 showed that there was a significant increase in 3xTg-AD-P021 compared to WT and 3xTg-AD-vh in the CA1 (Fig. 35a, e, p = 0.0038) and CA3 regions (Fig. 35a, e, p = 0.0010). In the DG (Fig. 35a, e, p = 0.0314) and parietal cortex (Fig. 35a, e, p = 0.0169) a significant difference was found between 3xTg-AD-vh and 3xTg-AD-P021. Similarly, Western blot analysis of the hippocampus showed a trend toward a decrease in the level of PDS-95 expression in the 3xTg-AD-vh mice compared to WT (Fig. 36b, c, p = 0.07) and a trend toward an increase in 3xTg-AD-P021 compared to 3xTg-AD-vh (Fig. 36b, c, p = 0.07). I found also that the NR1 expression in the CA3 region increased in 3xTg-AD-P021 compared to WT and 3xTg-AD-vh (Fig. 35c, e, p = 0.0091).

A trend toward a decrease was found in the MAP2 expression in the CA3 region (Fig. 35b, e, p = 0.09), DG (Fig. 35b, e, p = 0.0416) and the parietal cortex (Fig. 35c, e, p = 0.0392) in the 3xTg-AD-vh compared to WT. However, no apparent effect of P021 to prevent this deficit was detected. The analysis of the expression of synapsin 1 immunohistochemically showed a trend toward a decrease in the DG in the 3xTg-AD-vh compared to WT (Fig. 35d, e, p = 0.07). Similarly, no difference was detected by Western blots (Fig. 36a, c) and no apparent effect of P021 was detected either immunohistochemically or by Western blots. I analyzed the level and the expression of synaptophysin both immunohistochemically and biochemically and I found a significant decrease in 3xTg-AD-vh compared to WT by Western blots (Fig. 36a, c, p = 0.0339). However, I did not detect any effect of P021 on synaptophysin expression. I confirmed the genotype of 3xTg-AD-vh and 3xTg-AD-P021 mice with Western blots using the human-specific tau antibody 43D (Fig. 36a, b).
Fig. 35: Effect of P021 on synaptic deficit, 9 months post-treatment in 3xTg-AD mice.
a) Immunohistochemical analysis of the level of expression of a) PSD-95 in the CA1, CA3, DG, and parietal cortex b) MAP2 in the DG (upper panel), CA3 (middle panel), and parietal cortex (lower panel) c) NR1 in the CA3 region. d) Analysis of the synapsin 1 level of expression in the DG. e) quantification of immunohistochemical staining. One Way ANOVA post hoc test.
*p<0.05; **p<0.01; ***p<0.001. Scale bar = 100μm. (WT, n = 5; Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Quantification is shown as mean ± SD.
Fig. 36: Effect of P021 at 12 months of age in 3xTg-AD mice.
a) Analysis of the protein expression level in the cortex by Western blots. b) Analysis of the expression level of protein in the hippocampus and c) quantification of the blots. One Way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. (WT, n = 5; Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Quantification is shown as mean ± SD.

Effect of P021 on prevention of synaptic loss after 15 months of treatment

The analysis of the expression of MAP2 immunohistochemically showed that there was a trend toward a decrease in the CA1 between WT and 3xTg-AD-vh mice (Fig. 37a, f, p = 0.06). A significant effect of P021 was detected at this time point to prevent synaptic loss in the CA1
region (Fig. 37a, f, p = 0.0065). In the CA3 region a trend toward a significant decrease was detected in 3xTg-AD-vh as compared with WT mice (Fig. 37a, f, p = 0.09). P021 was found to rescue this deficit (Fig. 37a, f, p = 0.0226). In the DG (Fig. 37a, f, p = 0.0239) and the parietal cortex (Fig. 37a, f, p = 0.0491) a significant preventive effect of P021 was found in 3xTg-AD-P021 compared with 3xTg-AD-vh mice.

The analysis of the synapsin 1 expression immunohistochemically showed a decrease of the expression in 3xTg-AD-vh and no apparent effect of P021 treatment. Immunohistochemical analysis showed a significant decrease in the CA1 region (Fig. 37b, f, p = 0.0395), a trend toward a decrease in the CA3 region (Fig. 37b, f, p = 0.07) and trend toward a decrease in the parietal cortex (Fig. 37b, f, p = 0.06) in the 3xTg-AD-vh compared to WT.

Immunohistochemical analysis of the expression of GluR1 showed a significant increase in the 3xTg-AD-P021 mice in the CA1 region compared to the 3xTg-AD-vh (Fig. 37c, f, p = 0.0365).

Immunohistochemical investigation of the expression of synaptophysin in the CA1 region showed a significant increase in the 3xTg-AD-P021 compared to WT (Fig. 37d, f, p = 0.0450) and a trend toward an increase in the level of synaptophysin in the parietal cortex in 3xTg-AD-P021 compared to WT (Fig. 37d, f, p = 0.0623). The analysis of the expression of NR1 showed that it increased in the DG (Fig. 37e, f, p = 0.0306) and the parietal cortex (Fig. 37e, f, p = 0.0476) of the 3xTg-AD-P021 compared to WT.

Western blot analyses of synapsin1 level in the cortex showed a significant preventive effect of P021 (Fig. 38a, c, p = 0.0003) in 3xTg-AD-P021 mice. Similarly, I found an increase in the level of synaptophysin in the cortex of the 3xTg-AD-P021 mice compared to 3xTg-AD-vh (Fig. 38a, c, p = 0.0045). The NR1 expression level shows a trend toward an effect of P021 (Fig.
38a, c). In the hippocampus Western blot analysis showed a trend toward an effect of P021 in increasing the level of MAP2 and synapsin1 (Fig. 38b, c, p = 0.05 and p = 0.06, respectively). The 3xTg-AD-vh and 3xTg-AD-P021 genotypes were determined biochemically using the 43D antibody which recognizes only human tau (Fig. 38a, b).

Fig. 37: Preventive effects of P021 on synaptic deficits at 15 months post-treatment by immunohistochemistry in 3xTg-AD mice.
a) Investigation of the level of expression of MAP2 in the CA1, CA3, DG, and parietal cortex. b) Synapsin 1 level of expression in the CA1, CA3, and parietal cortex. c) Level of expression of GluR1 in the CA1 region. d) Level of expression of synaptophysin in the CA1 and the parietal cortex. e) Level of expression of NR1 in the DG and the parietal cortex and f) quantification of immunohistochemical staining. One Way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100μm. (WT, n = 5; Tg-AD-vh, n = 3; Tg-AD-P021, n = 5). Quantification is shown as mean ± SD.
Fig. 38: Preventive effect of P021 after 15 months post-treatment by Western blots in 3xTd-AD mice.

a) Analysis of protein expression in the cortex. b) Analysis of protein expression in the hippocampus and c) quantification of the blots. One Way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. (WT, n = 5; Tg-AD-vh, n = 3; Tg-AD-P021, n = 5). Quantification is shown as mean ± SD.

Effect of P021 on synaptic loss after 18 months of treatment

A significant increase of the synapsin 1 expression immunohistochemically in the CA1 region was found in the 3xTg-AD-P021 compared to WT (Fig. 39a, c, p = 0.0384) and a trend toward an increase compared to the 3xTg-AD-vh (Fig. 39a, c, p = 0.0791). The expression of
NR1 in the parietal cortex decreased in the 3xTg-AD-vh compared to WT (Fig. 39b, c, $p = 0.0305$) and a trend toward an increase in 3xTg-AD-P021 compared to 3xTg-AD-vh (Fig. 39b, c, $p = 0.05$) was found.

Western blot analysis showed a significant increase in the level of synaptophysin in the cortex (Fig. 39d, f, $p = 0.03$). In the hippocampus, synaptophysin showed a trend toward a decrease in the 3xTg-AD-vh compared to WT (Fig. 39e, f, $p = 0.07$). In the cortex, a trend toward a decrease in the ratio of pCreb and Creb was detected in the 3xTg-AD-vh compared to WT and 3xTg-AD-P021 (Fig. 39d, f). The MAP2 level showed a significant increase in the P021-treated group compared to 3xTg-AD-vh in the cortex (Fig. 39d, f). The level of PSD-95 in the hippocampus showed a trend toward an increase for the 3xTg-AD-P021 compared to the 3xTg-AD-vh (Fig. 39e, f, $p = 0.09$). The GluR1 expression showed a significant decrease in the 3xTg-AD-vh (Fig. 39e, f, $p = 0.02$) and a trend toward an effect of P021 (Fig. 39e, f, $p = 0.06$) in the hippocampus, no changes were detected in the cortex (Fig. 39d, f). The level of the ratio of pCreb and Creb showed a significant increase in the 3xTg-AD-P021 compared to the other two groups (Fig. 39e, f, $p = 0.0007$). The GluR2/3 level showed a significant increase in the WT compared to the other two groups (Fig. 39e, f, $p = 0.0002$).
Fig. 39: Preventive effect of P021 on synaptic deficit at 18 months post-treatment in 3xTg-AD mice.

a) Immunohistochemical analysis of synapsin 1 level in the CA1 region and b) NR1 level of expression in the parietal cortex. c) Immunohistochemical quantification. d) Western blot analysis of level of expression of synaptic proteins in the cortex and e) in the hippocampus and f) quantification of the blots. One way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 μm. (WT, n = 5; Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). Quantification is shown as mean ± SD.
6.2.2 Preventive effects of P021 on neurodegeneration

*Effect of P021 on prevention of neurodegeneration after 9 months of treatment in 3xTg-AD mice*

Neurodegeneration was analyzed using Fluorojade C staining and the data were analyzed as percent of area occupied by the degenerated neurons. The degenerated neurons were stained as bright green on a dark green background. The quantification of the percent of area occupied revealed that the area occupied by degenerated neurons was significantly higher in the 3xTg-AD-vh compared to WT and 3xTg-AD-P021 in the CA3 (Fig. 40a, d, p = 0.0066), DG (Fig. 40a, d, p < 0.0001), parietal (Fig. 40a, d, p = 0.0351), and frontal (Fig. 40a, d, p = 0.0121) cortices. P021 treatment for 9 months showed a complete rescue of the neurodegeneration.

*Preventive effect of P021 on neurodegeneration at 15 months post-treatment in 3xTg-AD mice*

Neurodegeneration was found to increase in the 3xTg-AD-vh compared to WT, and the P021 treatment for 15 months was able to prevent this impairment. These changes were seen in the CA1 (Fig. 40b, d, p = 0.0039), CA3 (Fig. 40b, d, p = 0.0431), DG (Fig. 40b, d, p = 0.0021), and parietal cortex (Fig. 40b, d, p = 0.0001).

*Preventive effect of P021 on neurodegeneration at 18 months post-treatment in 3xTg-AD mice*

At 21 months of age the percent of area occupied by the degenerated neurons was higher in 3xTg-AD-vh compared to WT, and P021 treatment was able to completely prevent this deficit in CA1 (Fig. 40c, d, p = 0.0011), CA3 (Fig. 40c, d, p = 0.0133), DG (Fig. 40c, d, p = 0.0013), and parietal cortex (Fig. 40c, d, p = 0.0056).
Fig. 40: Preventive effect of P021 on neurodegeneration in 3xTg-AD mice.
a) At 9 months post-treatment in the CA3, DG, parietal and frontal cortices (WT, n = 5, Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). b) At 15 months post-treatment in the CA1, CA3, DG, and parietal cortex (WT, n = 5, Tg-AD-vh, n = 3; Tg-AD-P021, n = 5). c) At 18 months post-treatment in the CA1, CA3, DG, and parietal cortex and (WT, n = 5, Tg-AD-vh, n = 5; Tg-AD-P021, n = 5). d) quantification of immunohistochemical staining. One way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 μm. Quantification is shown as mean ± SD.

6.2.3 Effect of P021 on boosting neurogenesis

The effect of P021 on boosting neurogenesis was investigated at 9 months post-treatment (12 month old 3xTg-AD) by immunohistochemical staining for Ki-67, a cell proliferation marker that measures early stages of the neurogenesis and DCX a marker for immature adult-born neurons whose expression levels reflect neurogenesis. As expected, I found that the number of
DCX+ cells was significantly reduced in the 3xTg-AD-vh compared to WT (Fig. 41a, c, p = 0.0009) and that P021 rescued this deficit (Fig. 41a, c, p < 0.0001). Remarkably, the level of neurogenesis was rescued to more than WT level (Fig. 41a, c, p < 0.001). Similarly, a decrease in the number of Ki-67+ cells was observed in the 3xTg-AD-vh mice compared to WT (Fig. 41b, c, p = 0.0057) and it was rescued with the P021 treatment (Fig. 41b, c, p < 0.0001).

**Fig. 41: Preventive effects of P021 on neurogenesis in DG at 9 months post-treatment in 3xTg-AD mice.**

a) DCX and TOPRO staining in WT (left panel), 3xTg-AD-vh (middle panel), and Tg-AD-P021 (right panel). b) Ki-67 and TOPRO staining in WT (left panel), 3xTg-AD-vh (middle panel), and Tg-AD-P021 (right panel). c) Densiometric quantification of the number of DCX+ and Ki-67+ cells. Arrow heads indicate positive cells. One way ANOVA post hoc test. *p<0.05; **p<0.01; ***p<0.001. Scale bar = 100 μm. Quantification is shown as mean ± SD.
6.3 Discussion

Since synaptic and neuronal losses occur very early in the disease process and are the best correlates of cognitive impairment and dementia in AD, I postulated that an efficient treatment for AD would be one that starts early in the disease process and is able to achieve synaptoprotection and neuroprotection and thus halt the disease progression. I further postulated that a treatment that is initiated during the period of synaptic compensation would make this phenomenon successful and help slow down synaptic and neuronal loss as well as boosts neurogenesis and consequently cognitive deterioration. Herein, I applied such a strategy: I administered the P021 in diet during the synaptic compensation period and I followed its effect at 9, 15, and 18 months post-treatment.

In the present study I found that at 3 months of age, before the start of the P021 treatment, there was a synaptic loss, as seen by the decrease in the level of MAP2, synaptophysin, PSD-95, NR1, and the ratio of pCreb and Creb. At 9 months post-treatment I found a synaptic deficit in the 3xTg-AD-vh mice compared to the WT, as shown by a decrease in the level of MAP2 and synapsin 1. While I found a rescue of synaptic deficit post-9 month treatment as seen immunohistochemically with PSD-95 and NR1, the strongest effect of P021 on rescuing synaptic deficit was at 15 months post-treatment on the MAP2, synaptophysin, synapsin 1, GluR1 and NR1 expression levels as shown immunohistochemically and biochemically. At 18 months post-treatment P021 was able to prevent synaptic deficit, as shown by immunohistochemistry and Western blots.

Our data showed that at exactly 3 months of age, before I started the treatment with P021, there was synaptic loss, as shown by a decrease in the synaptophysin, MAP2, PSD-95 and NR1 expression levels, the ratio of pCreb and Creb, as well as a trend toward a decrease in the GluR1 level in the 3xTg-AD mice compared to the WT. These data further corroborate our findings.
described in Chapter 3. An early drop in the synaptophysin level of expression is also known in early AD patients and this decrease is correlated with early cognitive impairment and the severity of dementia (Heffernan et al., 1998, Masliah et al., 2001, Callahan et al., 2002). Similarly, I found here and in Chapter 3 an early reduction in the synaptophysin level as well as cognitive impairment in the 3xTg-AD mice.

At 9 months post-treatment the analysis of the expression level of PSD-95 immunohistochemically showed that P021 treatment was able to rescue the decrease in the expression in 3xTg-AD mice in the CA1, CA3, DG and parietal cortex. Similarly, with Western blots I found a trend toward a decrease in the level of PSD-95 in the 3xTg-AD-vh and a trend toward an effect of P021 in the hippocampus. The expression of NR1 decreased also in the 3xTg-AD-vh in the parietal cortex and a trend toward an effect of P021 was seen. I found a synaptic deficit, as seen in the drop of MAP2 expression in the DG, CA3, and parietal cortex and the synapsin 1 expression in the DG in 3xTg-AD mice. These data showed that P021 at 12 months of age was effective in restoring synaptic loss, as seen in a restored expression level of PSD-95 and NR1. Our immunohistochemical findings were further corroborated with the Western blot analysis of the cortex.

At 15 months post-treatment P021 showed the strongest effect on preventing synaptic loss. This may be because at 18 months of age the 3xTg-AD-vh mouse brain has already undergone extensive synaptic loss so the effect of P021 becomes quite apparent. Indeed, P021 rescued the decrease in MAP2 expression in all the brain regions analyzed immunohistochemically (CA1, CA3, DG and parietal cortex). Synaptophysin loss was also prevented in the CA1 and the parietal cortex. A decrease in the GluR1 expression was also prevented in the CA1 region of the hippocampus. Western blot analysis of the protein level of
synaptophysin further corroborated our data. Similar data was reported in other P021 studies (Blanchard et al., 2010a, Blanchard et al., 2010b, Li et al., 2010, Blanchard et al., 2011, Chohan et al., 2011, Bolognin et al., 2012, Kazim et al., 2014). The preventive effect of P021 on rescuing synapsin 1 expression was seen by Western blot analysis. This strong effect of P021 should be interpreted in light of our previous data on Aβ and tau pathologies and cognition. I showed previously (Chapter 4) that there was a strong effect of P021 on preventing cognitive impairment and Aβ and tau pathologies (Chapter 5), thus P021 would be expected to prevent synaptic pathology as well.

At 18 months post-treatment an effect of P021 on rescuing the synapsin1 expression was seen immunohistochemically in the CA1 region of the hippocampus. A trend toward a rescuing effect of P021 on the expression of NR1 in the parietal cortex was found. Western blot analysis of the cortex and the hippocampus revealed a preventive effect of P021 on rescuing the level of synaptophysin and MAP2 in the cortex and the ratio of pCreb and Creb in the hippocampus. A trend toward an effect of P021 on rescuing PSD-95 and GluR1 expression was also found in the hippocampus. Thus, collectively, our data show that early chronic treatment with P021 can be effective even until later age in rescuing neuronal plasticity deficits in 3xTg-AD mice.

In the present study I found that the different brain regions of the 3xTg-AD-vh mice had undergone massive degeneration compared to WT mice, and P021 treatment was able to rescue this impairment at 12, 18 and 21 months of age.

The level of neurogenesis in the present study was investigated by the quantification of the number of DCX+ cells and Ki-67+ cells in the DG area at 9 month post-treatment with P021. My results show that neurogenesis was defective in 3xTg-AD-vh mice and that the treatment with P021 was able to rescue this deficit. This neurogenic effect of P021 could be through the
inhibition of the LIF signaling pathway or the increase in the BDNF expression. My results are in agreement with what was shown previously when P021 was injected subcutaneously to normal adult C57Bl6/J mice (Li, et., 2010) or by oral gavage to a rat model of cognitive aging (Bolognin et al., 2014).

6.4 Conclusion
My data showed that there was a synaptic loss in 3 month old 3xTg-AD mice. After 9, 15, and 18 months of treatment, P021 was able to prevent synaptic loss. Furthermore, neurodegeneration analysis using Fluorojade C staining showed that P021 completely prevented neurodegeneration at the three different time points. At 12 month of age (9 month post-treatment) P021 was also able to rescue the deficit in neurogenesis. These data showed that P021 could be a potential drug to restore synaptic loss, boost neurogenesis and prevent neurodegeneration.
Chapter 7. Discussion

Taken together, the results of the present project demonstrate for the first time that the administration of the neurogenic/neurotrophic peptidergic compound P021 at a very early stage of the disease can prevent synaptic and neurogenesis deficit, cognitive impairment and Aβ and tau pathologies in 3xTg-AD mice. Behavioral analysis of 3xTg-AD mice with the Morris Water Maze task showed that the 3xTg-AD mice were already cognitively impaired prior to the initiation of the treatment with P021. Impairment in cognition was accompanied by a decrease in the level of several synaptic markers such as synaptophysin and compensation at later time points. The P021 provided during this compensation period showed that it can prevent synaptic loss, neurogenesis deficit, cognitive impairment and Aβ and tau pathologies. Most importantly, the analysis across time of the effect of P021 on Aβ and tau pathologies demonstrated a strong effect on slowing down the progress of Aβ and tau pathologies, as shown by a decrease in the 4G8 and AT8 load at 9 months, 15 months and 18 months post-treatment. Furthermore, I unexpectedly found that P021 was able to increase the survival rate in the 3xTg-AD to 87% compared to only 41% for 3xTg-AD-vh. Analysis of body weight and the food intake showed that P021 had no apparent side effects.

7.1 Synaptic compensation
I found that at 12-14 weeks of age the 3xTg-AD mice were impaired. The immunohistochemical and biochemical analysis of the brain tissues of 3xTg-AD mice from 10, 12, 13, 14, 15, and 16 weeks of age demonstrated the level and the expression of several synaptic markers, most importantly synaptophysin, dropped sharply at 12 weeks, then came back almost to the level of normal WT from 13 to 16 weeks because of the synaptic compensation
phenomenon. The synaptic compensation phenomenon was reported previously in AD patients at very early stages of the disease (Counts et al., 2006) and in cognitive aging (Bertoni-Freddari et al., 1988, Barnes, 1994, Brown et al., 1998). My data further showed that when I compared the expression levels of synaptic markers in the 3xTg-AD to those of WT the compensation appeared to be region specific and time specific. Similarly, synaptic compensation in cognitive aging was shown to be region specific and it was suggested to account for the spared cognition in elderly people (Bertoni-Freddari et al., 1988, Barnes, 1994). In AD, however, synaptic compensation seems to be unsuccessful, which is why cognitive deterioration occurs. This is in agreement with our finding that the 3xTg-AD mice were impaired in the presence of the compensation phenomenon. It could be suggested, however, that synaptic compensation in humans is what accounts for a relatively long period of MCI in which cognitive deterioration and neuronal and synaptic loss happen very slowly for up to 10 years. Likewise, in the 3xTg-AD mice mutations are carried in their genes from embryonic day 11 but the appearance of the Aβ and tau pathologies starts only at around 9 months of age for Aβ and 12 months for tau. Several reports in the literature emphasize the importance of the compensation phenomenon at the level of the synapse and at the level of the new born neurons to boost the efficiency of the therapeutic intervention. Thus a drug that could be provided during this period would help the brain to counteract the disease progression.

7.2. Effect of P021 on cognitive impairment and survival rate

Previously, many attempts were made to use neurotrophic factors as therapeutic agents for AD. However, most of them failed because of their short half-life, poor BBB permeability and several side effects such as loss of appetite, weight loss, muscle pain, anorexia and hyperplasia (Saragovi and Gehring, 2000). All of these side effects hinder the use of
neurotrophic factors in Alzheimer’s therapy. To overcome these problems, several studies shift to peptide mimetics, which are derived from the most active region of the neurotrophic factors. Since they are very small peptides they have a better ability to cross the BBB, better half-life and lesser side effects. The same approach was used in this study. I used P021, which is derived from the most active region of the CNTF. We then added the adamantane group to increase its lipophilicity and make it BBB permeable and decreased its degradation with peptidases (Li et al., 2010). We showed previously that P021 has no apparent side effects (Blanchard et al., 2010b, Kazim et al., 2014).

The present study is solely preventive; My aim was to provide P021 in the mouse diet very early at 3 months of age, during the period of synaptic compensation and before the onset of Aβ and tau pathologies, and most importantly before severe synaptic or neuronal loss. The chronic administration of P021 in this study from 3 months to 21 months of age showed that P021 was able to prevent cognitive impairment completely in the mild and severe stages of the AD-like pathological progress in 3xTg-AD mice. I showed that at 12 months of age, after treatment with P021 for 9 months, spatial reference memory impairment was prevented in the 3xTg-AD-P021. Furthermore, at 18-19 months of age, after 15-16 months of P021 treatment, I showed that short-term spatial memory impairment was prevented in the 3xTg-AD-P021, as seen in a better performance in the object location task compared to 3xTg-AD-vh. Impairment in episodic memory was prevented in the object recognition task in the 3xTg-AD-P021 while both the 3xTg-AD-vh and WT were impaired. The WT mice were impaired because of the effect of aging and this was further corroborated from the literature (Davis et al., 2013). Since spatial navigation and episodic memories are severely affected in AD at early stages and I showed in this study that P021 was able to prevent the impairment, this shows its potential therapeutic
effects in AD. Cognitive impairment followed by dementia is one of the major clinical symptoms that render AD patients totally dependent on others and bedridden at later stages of their life, so if I can prevent cognitive deterioration I will be able to halt or slow down the disease process. Not only was early treatment with P021 able to prevent the onset of cognitive impairment, but also I unexpectedly found that it increased the survival rate of the 3xTg-AD-P021 compared to 3xTg-AD-vh. It is well documented in the literature that AD decreases the survival rate of patients. For instance, over 14 years the survival rate of AD patient is only 2.4% versus an expected rate of 16.6% (Molsa et al., 1995). In our study, the 3xTg-AD-P021 survival rate was 87% compared to only 41% in 3xTg-AD-vh. This shows that delaying the onset of cognitive impairment and the other pathologies in AD patients would help to increase their survival rate.

7.3 Effect of P021 on Aβ and tau pathologies

Aβ and tau pathologies are the two major hallmarks of AD. Cognitive impairment is known to correlate with NFT counts but not with Aβ plaques (Arriagada et al., 1992a, Gomez-Isla et al., 1997). The spatial distribution of tau pathology follows a stereotypic pattern that starts in the limbic system and then spreads to the neocortical regions. Since the limbic regions are the primary targets of NFT pathology and they are known for their important role in memory it is reasonable to expect a correlation between tau pathology and cognitive impairment. Since tau pathology correlates with cognitive impairment its prevention could help to stop cognitive deterioration. Indeed, in my study I found that early treatment with P021 in 3xTg-AD mice was able to slow down the progression of Aβ and tau pathologies across time from the time of their onset until the progression to severe stages. Slowing down the pathologies could be the biological correlate to slowing down cognitive deterioration and the rescue of cognitive impairment seen in my study at different time points. My data are in agreement with reports in
the literature that the use of plasma rich growth factors is able to decrease Aβ deposition, tau phosphorylation and cognitive impairment in APP/PS1 mice (Anitua et al., 2014).

### 7.4 Effect of P021 on neurodegeneration and neuroregeneration

Synaptic deficit is widely reported to correlate with cognitive impairment (DeKosky and Scheff, 1990, Terry et al., 1991). Furthermore, it has been shown that synaptic loss correlates with the increase in NFT counts. Synaptic loss was found to follow a similar pattern to NFT pathology, in which neurons in the entorhinal cortex are first affected and then the hippocampus (Coleman et al., 2004). The hippocampus in AD has been shown to have the greatest synaptic loss, from 44% to 55% (Scheff et al., 2007). Early intervention within the onset of synaptic loss or before was suggested to be effective on preventing and slowing down the progression of the neuropathological features of AD. In this study P021 was provided in food diet just at the onset of synaptic loss for nearly the whole mouse lifespan. I found that P021 was able to prevent synaptic loss, especially at 18 and 21 months of age. Our data are in agreement with our previous studies using P021 in AD mouse models (Blanchard et al., 2011, Bolognin et al., 2012, Kazim et al., 2014) as well as other animal models of neurodegenerative diseases (Blanchard et al., 2011) and cognitive aging (Bolognin et al., 2014). Similarly, P021 treatment was able to prevent neurodegeneration at the three time points.

### 7.5 Effect of P021 on neurogenesis

Since it is well established that neurogenesis contributes to information storage and retrieval as well as the plasticity of the hippocampus, its disruption most likely will affect memory and cognitive abilities. Indeed, in AD neurogenesis is disrupted very early in the disease process. The impaired neurogenesis in AD was shown to be due to an imbalance of neurotrophic factors in the brain biochemical milieu with a decrease in the BDNF and NT-4 expression levels
and an increase in the expression level of FGF-2. We previously reported an increase in neurogenesis in AD postmortem brains in the DG (Li et al., 2008). However, the self-repair attempt of the brain remains unsuccessful as the newly born neurons cannot make it to become fully mature and integrate into the surviving circuitry in AD brains. I thought that providing the brain with the trophic support would help the brain to compensate for the deficit in neurogenesis. In this study the effect of P021 to boost neurogenesis was studied at 9 month-post-treatment in the DG area. I found that neurogenesis was defective in the 3xTg-AD-vh compared to WT and that P021 was able to prevent this deficit and boost neurogenesis to a level more than WT. This was seen by the increase in the number of the DCX+ and Ki-67+ cells in the P021 treated group in the DG. The fact that neurogenesis decreased in the 3xTg-AD-vh mice was in agreement with what was found previously using this mouse model (Hamilton et al., 2010).

7.6 Possible mechanism of prevention of cognitive impairment and pathological features with P021

Previous investigation of the possible mechanisms of P021 showed that P021 acts by competitively inhibiting leukemia inhibitory factor (LIF) signaling. In neuronal cell culture P021 was shown to weakly inhibit LIF signaling through a slight inhibition of the phosphorylation of STAT3 in a dose dependent manner (Li et al., 2010). Furthermore, we showed previously that P021 acts by increasing the expression level of BDNF. Together the inhibition of LIF signaling and the increase in BDNF expression would inhibit stem cell proliferation and promote their maturation (Fig. 42a). Furthermore, BDNF has been shown to increase synaptic plasticity, neurogenesis and cell survival. The effect of P021 on preventing synaptic plasticity and cognitive impairment that I found here is probably due in part to the increase in BDNF expression. It is well established in the literature that an increase in BDNF expression leads to an increase in the
phosphorylation of GSK3β on its Serine 9 site, which is inhibitory. The prevention of Aβ and tau pathologies with P021 in this study may be through the inhibition of GSK3β signaling (Fig. 42b). Indeed, it has been shown that GSK-3β is a major tau kinase (Kosik, 1992) and its overexpression leads to hyperphosphorylation of tau and microtubule disruption in mice (Lucas et al., 2001). In AD, GSK3β is known to mediate Aβ induced neuritic damage (DaRocha-Souto et al., 2012). Moreover, the GSK 3α isoform is known to increase APP processing (Phiel et al., 2003) and Aβ production. So inactivation of GSK3β would result in a reduction of the Aβ load and tau hyperphosphorylation, as seen in the present study. In the neurogenic niches hyperactivation of GSK3β leads to impaired neurogenesis in mice. Thus, by decreasing GSK3β activity, neurogenesis can be rescued. Since it has been shown that GSK3β promotes apoptosis, its inhibition also would lead to the inhibition of neurodegeneration (Beurel et al., 2010).
Fig. 42: Mechanism of action of P021. a) Neurogenic/ neurotrophic effect of P021. b) Mechanism of inhibition of Aβ and tau pathologies by P021. (Kazim et al, Neurbiol. Dis., 2014).
7.7 Future directions

My findings (chapter 3) suggest that in 3xTg-AD mice the brain attempts to self-repair by the synaptic compensation phenomenon as a reaction to the early synaptic loss. However, even in the presence of synaptic compensation this attempt is unsuccessful and does not prevent cognitive impairment, as seen in human cognitive aging. Future work should then focus on answering the question of what is lacking in this compensation. Is it insufficient or is it abnormal so that it cannot reverse cognitive impairment? An approach to answer this question would be to investigate the morphology of the spines with the lipophilic staining DiI to determine if they have a normal shape or large and stubby spines that are pathological. Since it is reported that in AD patients the compensation in the surviving synapses happens by increasing the synapse size so that the total contact surface area is preserved, a measure of the size of the synapses could be another approach to investigate in future studies. Future work could also focus on determining whether the number of synapses really increased, causing the observed increase in synaptic markers, or if it was simply a result of an increase in synaptic size. Furthermore, I could investigate the electrophysiological component of this compensation. For example, I could record from a single cell with a patch clamp method to detect an increase in the level of the excitatory post-synaptic potential. Then I could study it at the level of the neuronal network. If there is an increase in the amplitude of neuronal firing would this result in an increase in the whole neuronal firing? Would an increase in neuronal firing result in a compensation for the whole neuronal network or disrupt it? If the latter was the case, instead of compensating for the synaptic loss compensation would become the driving force for synaptic loss and neurodegeneration.

Since our study is of secondary prevention because it was initiated after the onset of cognitive impairment and synaptic loss, future directions would be to start the treatment of mice
in utero by providing the drug in the food to the mothers from conception until weaning and then later on to pups after weaning. Since the expression of the mutated transgenes in 3xTg-AD mice starts from embryonic day 11 and since an early treatment before the onset of synaptic loss and cognitive impairment would be more effective, I can consider the data from our study as encouraging the start of treatment in utero. Considering all ages together, P021 was able to decrease the Aβ and tau loads and it was able to prevent cognitive impairment as well as synaptic loss. I expect that in utero treatment would be stronger in preventing the Aβ and tau pathologies as well as synaptic loss. This study would be of great importance to human preclinical trials, especially those of the familial form of the disease. If toxicological testing shows that P021 is safe to use for humans from the embryonic stage then I could prevent AD in people who carry the mutated genes in their genome and are destined to develop it later on in their life.

7.8 Conclusion

Together the results of this study showed for the first time that secondary prevention using P021 in 3xTg-AD mice is able to prevent cognitive impairment, Aβ and tau pathologies and synaptic and neuronal loss and can boost neurogenesis. Taking advantage of the synaptic compensation phenomenon and the ability of the brain to self-repair very early in the disease process I was able to show that P021 treatment was able to prevent cognitive impairment at three different time points at 9, 15-16, and 16-17 months post-treatment. Most importantly I showed that P021 treatment increases the survival rate of 3xTg-AD mice and can prevent Aβ and tau pathologies. Lastly I found that P021 was able to prevent synaptic loss by increasing the level and the expression of different synaptic markers as I showed biochemically as well as immunohistochemically at 9, 15, and 18 months post-treatment. Thus, together these data
suggest P021 as a potential drug for AD treatment in future prevention trials, since prevention trials now use only anti-Aβ therapy which, to date, has proven in human clinical trials to be ineffective.
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