Prevention and treatment: Effects on pathological alterations in transgenic Alzheimer's disease mouse models

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I hereby declare that my doctoral thesis entitled “Prevention and treatment: Effects on pathological alterations in transgenic Alzheimer’s disease mouse models” has been written independently with no other sources and aids than quoted.

Martina Stazi,

Göttingen, March 2021

"Per aspera sic itur ad astra "

Cicerone

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Wirths O, Walter S, Kraus I, Klafki HW, **Stazi M** et al. N-truncated $A\beta_{4-x}$ peptides in sporadic Alzheimer's disease cases and transgenic Alzheimer mouse models. Alzheimer's Research & Therapy. 2017;9(1):80. doi:10.1186/s13195-017-0309-z. PMID: 28978359

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder representing the most common form of dementia worldwide. To date, no successful therapeutic approach to treat AD has been developed, hence preventive strategies started to become a major research focus.

In this work, the effects of pharmacological treatment strategies such as chronic memantine, caffeine or riluzole supplementation, as well as the effect of a stimulating environmental living condition on the pathological alterations have been examined in Tg4-42^{hom} and 5xFAD mice, representing two different AD mouse models.

The Tg4-42 model overexpresses only the Aβ_{4-42} peptide mainly in the hippocampus, lacking any mutations linked to the familial form of the disease. Although this mouse model does not present overt extracellular Aβ plaques, Aβ_{4-42} forms neurotoxic aggregates that well correlate with the age- and dose-dependent pyramidal neuron loss in the CA1 region of the hippocampus as well as with robust learning and memory deficits. It could be shown that long-term oral treatment with either memantine, caffeine or riluzole completely rescued behavioural deficits in 6-month-old Tg4-42^{hom} mice. Interestingly, these beneficial effects on learning and memory were accompanied by a significant amelioration of neuronal loss and a robust increase in neurogenesis. The present study presents evidence that long-term oral treatment with these drugs prevents behavioural decline as well as neuron loss and impaired neurogenesis in a mouse model reflecting the sporadic form of AD.

Recently, epidemiological data revealed a potential protective role of physical exercise and cognitive stimulation on AD risk. Hence, it could be demonstrated in Tg4-42^{hom} mice that living in a stimulating environment not only improves general behaviour but also ameliorates sensory-motor deficits. We have shown that the housing condition exerts a strong beneficial effect on a typical floating phenotype in Tg4-42^{hom} mice, since none of the animals housed in an enriched environment presented that characteristic.

The 5xFAD model is a well-characterized and commonly studied AD mouse model, reflecting first and foremost the familial form of the disease. In the present work, it could be demonstrated that, despite of an amelioration of memory deficits, chronic caffeine intake has no major influence on the typical pathophysiological alterations in the 5xFAD model, since neither changes with regard to Aβ plaque deposition, neuroinflammation, $A\beta_{1-42}$ levels or APP processing were observed. These results suggest that a therapeutic intervention, such as chronic caffeine administration, might have a crucial impact on cognition without influencing the aggressive AD pathology observed in 5xFAD mice after onset of pathology.

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LIST OF ABBREVIATIONS

Abbreviations	Description
pTau	Hyper-phosphorylated tau protein
ABC	Avidin-biotin complex
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ADE	Aβ-degrading enzyme
AICD	Amyloid precursor protein intracellular domain
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
BACE1	β-site cleaving enzyme 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA1/2	Cornu ammonis area 1/2
CaMKII	Calcium calmodulin-dependent kinase II
CNS	Central nervous system
Co	Cortex
CR	Cognitive reserve
CREB	Cyclic AMP response element binding protein
CSF	Cerebrospinal fluid
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
ddH_2O	Double-distilled water
DG	Dentate gyrus
DI	Discrimination index
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
e.g.	Exempli gratia
EAAT	Excitatory amino acid transporter
EE	Environmental enrichment
ELISA	Enzyme-linked immunosorbent assay
EO-FAD	Early-onset familial Alzheimer's disease
EPM	Elevated plus maze
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
EtOH	Ethanol

F	Fimbria
FCS	Fetal calf serum
FDA	Food & Drug Administration
FELASA	Federation of European Laboratory Animal Science Association
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthasekinase 3β
GWAS	Genome-wide association studies
H	Hilus
i.p	Intraperitoneal
JNK	c-Jun N-terminal-kinase
KA	Kainate
LAVES	Landesamt für Verbraucherschutz und Lebensmittelsicherheit
LH	Left hemisphere
LOAD	Late-onset of Alzheimer's Disease
LTP	Long Term Potentiation
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
MWM	Morris water maze
NFTs	Neurofibrillary tangles
NMDANR2A	N-methyl-D-aspartate NR2A subunit
NMDANR2B	N-methyl-D-aspartate NR2B subunit
NOL	Novel object location
NOR	Novel object recognition
Ø	Diameter
O.N.	Over-night
O_1/O_2	Object
OCT	Optimal cutting temperature embedding medium
OF	Open field
p38-MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pCREB	Phosphorylated cyclicAMP response element binding protein
PDGF	Platelet derived growth factor-β
PFA	Paraformaldehyde
PHFs	Paired helical filaments
PSEN 1/2	Presenilin 1/2
r.p.m	Revolutions per minute
RH	Right hemisphere
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature

sAPP	Soluble amyloid precursor protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SG	Stratum granulosum
SGZ	sub-granular zone
SH	Standard housing
SL	Stratum lucidum
SLM	Stratum lacunosum-moleculare
SM	Stratum moleculare
SO	Stratum oriens
SP	Stratum pyramidale
SR	Stratum radiatum
Subi	Subiculum
SVZ	sub-ventricular zone
TBE buffer	Tris/borate/EDTA buffer
TBI	Traumatic brain injury
TBS buffer	Tris-buffered saline
Thal	Thalamus
TREM2	Triggering receptor expressed on myeloid cells 2
TRH	Thyrotropin-releasing hormone
TrkB	Tropomyosin receptor kinase B
VGlut	Vesicular glutamate transporter
WT	Wild-type
α2M	α2-macroglobulin
α7-nAchR	α-7 nicotinic acetylcholine receptor
αCTF/C83	α-C-terminal fragment
β-CTF/C99	β-C-terminal fragment

1. INTRODUCTION

1.1. Alzheimer's disease

Alzheimer's disease (AD) is an irreversible, chronic, and progressive neurodegenerative disorder representing more than 80% of dementia cases worldwide (Turner, 2005). Nowadays, it is estimated that more than 50 million people worldwide have dementia. It is predicted that the total estimated prevalence of AD is expected to be over 150 million by 2050 (Alzheimer's disease facts and figures, 2020). AD was first described by Alois Alzheimer in 1906 who outlined clinical and neuropathological characteristics such as progressive memory, language and sleep impairments, mood changes, as well as peculiar amyloid plaques and neurofibrillary tangles in the brain of the patients (Hippius and Neundörfer, 2003).

1.2. Risk factors of Alzheimer's disease

1.2.1. Genetic risk factors for Alzheimer's disease

Alzheimer's disease is a multifactorial and heterogeneous disease involving several different etiopathogenic mechanisms (Iqbal and Grundke-Iqbal, 2010). Two forms of AD have been recognized, which are based on genetic predisposition and on the age of disease onset. Less than 1-5% of AD cases are represented by the early-onset familial form (EO-FAD), which is caused by mutations in three different genes: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) (Zetterberg and Mattsson, 2014). The sporadic form accounts for the majority (~95-99%) of all diagnosed cases (Bekris et al., 2010) and is usually linked to a late-onset of AD (LOAD), meaning the disease manifests beyond the age of 65 years (Blennow et al., 2006). No known mutations are involved in the sporadic form of AD, however, genome-wide association studies (GWAS) reported more than twenty AD

susceptibility loci in European patients, with *APOE* (apolipoprotein E) representing the most significant risk factor (Karch et al., 2014). The *APOE* gene has three allele variants *APOE2*, *APOE3* and *APOE4*. The first one, *APOE2*, present in ~7% of the whole population, is assumed to be protective against AD. The *APOE3* allele is the most widely represented variant (~79%) and it seems that it does not have a major influence on AD risk (Mamun et al., 2020a). Conversely, the presence of one or two APOE ε4 alleles increases the disease risk by 3 and 8 times respectively (Corder et al., 1993; Martins et al., 2005). Moreover, two non-synonymous mutations, Q170H and R181G were identified in seven LOAD families in the *ADAM10* gene. ADAM10 belongs to the a disintegrin and metalloprotease (ADAM) family that has α-secretase activity (Allinson et al., 2003). It was reported that over-expression of the two *ADAM10* mutations *in vitro* lead to an increase of amyloid beta (Aβ) levels (Kim et al., 2009). Moreover, the same mutations decreased the α-secretase activity shifting the APP processing toward the amyloidogenic pathway in the TG2576 mouse model of AD (Suh et al., 2013). Next to *APOE ε4* and *ADAM10*, the GWAS discovered also a missense mutation, rs75932628, in the *TREM2* (triggering receptor expressed on myeloid cells 2) gene (Guerreiro et al., 2013; Jonsson et al., 2013), causing an increased predisposition risk of AD, since TREM2 is a key protein regulating microglia activity and neuroinflammation in AD brain (Mamun et al., 2020b).

1.2.2. Modifiable risk factors for Alzheimer's disease

The lack of knowledge about sporadic AD aetiology makes it very complex to prevent its onset and detect risk factors. Although prevention of modifiable risk factors may not be able to completely prevent future development of the disease, Baumgart and colleagues hypothesized that good lifestyle habits might contribute to diminish the risk of dementia

(Baumgart et al., 2015). Therefore, identifying potential modifiable risk factors may reduce the number of people affected by AD (Edwards et al., 2019).

One of the first hypothesis for the origin of AD is the so-called vascular hypothesis of AD. Changes in the vascular system result in an overall reduced cerebral perfusion leading to general brain dysfunction and cognitive deficits (Iadecola, 2013). Vascular risk factors are associated with the progression of dementia from mild cognitive impairment (MCI) to the severe stage of AD (Li et al., 2011; Luchsinger et al., 2005). Epidemiological evidence has shown that vascular disease and AD have different risk factors in common (Panpalli Ates et al., 2016). Among these are diabetes, hypertension, atrial fibrillation, APOE genotype, hypercholesterolemia, and atherosclerosis (Vijayan and Reddy, 2016). Hypertension and heart diseases such as cardiac arrest, arrhythmias, or atrial fibrillation are phenomena causing a reduction of blood and oxygen supply to the brain. This deprivation leads to neuronal loss (Kwok et al., 2011), with the consequence of brain failure associated with cognitive decline (Alosco et al., 2013). The association between heart failure and increased prevalence of AD and dementia is supported by the fact that hypo-perfusion induces brain hypoxia and neuronal damage (Muqtadar et al., 2012). Therefore, a history of heart and vascular system diseases might be considered risk factors for developing AD.

Atherosclerosis is also linked to an increased risk of developing AD, as much more severe atherosclerosis disease has been reported in AD compared to age-matched healthy individuals (Roher et al., 2003). This pathological condition diminishes brain blood flow and is associated with an increment in amyloid plaque load and more advanced Braak stage in AD patients (Beach et al., 2007).

Hypercholesterolemia has been suggested to be a high-risk factor for AD. Excessive levels of cholesterol have been associated with elevated Aβ production, reduced Aβ degradation and progression in AD neurodegeneration (Barbero-Camps et al., 2018). The Aβ peptide is

believed to represent one of the main causes of AD and details regarding its role will be discussed in the section 1.5.1.

The prevalence of diabetes and AD is increasing (Brookmeyer et al., 2018; Wang et al., 2018; Weuve et al., 2015). There are various hints suggesting that hyperglycaemia can cause a decline in cognitive function (Overman et al., 2017) and that diabetes might represent a possible risk factor for the progression of MCI and AD (E. González-Reyes et al., 2016; Gaspar et al., 2016; Pruzin et al., 2018; Rojas-Carranza et al., 2018). Different studies demonstrated that hyperglycaemia causes neurodegeneration due to an increase of Aβ accumulation (Macauley et al., 2015), an aggravation of oxidative stress (Gaspar et al., 2016), neuroinflammation (Rom et al., 2019), mitochondrial dysfunction (Silzer and Phillips, 2018), and overall neuronal damage in the brain (Kim et al., 2016).

A recent study has hypothesized that traumatic brain injury (TBI) might be an additional strong factor that leads to AD or dementia (Edwards et al., 2017). The impact of a TBI event causes direct damage to the brain. In particular, the TBI leads to a rapid necrosis of the tissue, oedema, increased intracranial pressure, and ischemia. These events are suggested to cause mitochondrial dysfunction, inflammation, oxidative stress, axonal degeneration or neuronal death, ultimately instigating cognitive deficits (Breunig et al., 2013; Gentleman et al., 2004). The neuronal loss observed after a TBI event involves the medial temporal lobe structures such as hippocampus and results in an increased ventricular volume (Blennow et al., 2016). Since TBI has in common different mechanisms of AD pathology, the neurodegenerative process triggered by TBI might provoke the progression of cognitive impairments that might later lead to AD. The risk of developing AD correlates with TBI severity (Graves et al., 1990; Guo et al., 2007). After a TBI accident, dementia diagnosis was increased by ~80% within a 15 years follow-up period (Nordström and Nordström, 2018). In a cohort study, the general risk of dementia in people without a history of TBI was 24% less high respect the ones with at least one event of TBI in life (Fann et al., 2018). Moreover, analysis of *post-*

mortem brains from TBI patients, showed evidence for higher Aβ plaque densities compared to age-matched controls without brain injury (Johnson et al., 2012). In addition, multiple TBI events are associated with tauopathies and TBI patients displayed increased tau levels in the cerebrospinal fluid (CSF) up to over 1000-fold compared to healthy individuals and neurological-diseased individuals of other origin (Zemlan et al., 1999).

1.2.3. Lifestyle

Clinical studies demonstrate that physical exercise might represent an important neuroprotective factor by maintaining cognition and brain neuroplasticity (Kramer et al., 1999; Winter et al., 2007). Moreover, physical activity seems to improve clinical symptoms of AD. Different studies have shown how aerobic exercise improved cognitive and memory performance in aging, MCI, and AD patients (Baker et al., 2010; Morris et al., 2017; Vidoni et al., 2012). Although a plethora of reports suggests that having an active lifestyle might be efficient to diminish the symptoms associated with AD in patients, there are no human studies describing its impact on e.g. Aβ deposition. To better understand this beneficial effect of exercise, several studies have been done in AD models. In transgenic mouse models of AD, physical exercise ameliorates behavioural deficits, reduces Aβ deposition and diminished apoptosis (Adlard et al., 2005; Liu et al., 2013; Nichol et al., 2007; Um et al., 2008). Moreover, in AD rodent models showing tau pathology, physical exercise was able to reduce the levels of total/phospho and insoluble tau (Belarbi et al., 2011; Leem et al., 2009; Ohia-Nwoko et al., 2014). One of the major factors that seems to be involved in this beneficial effect is the brain-derived neurotrophic factor (BDNF). In fact, after an acute exercise session, increased BDNF levels in the brain could be measured (Ferris et al., 2007; Neeper et al., 1995). The positive effect of BDNF in AD models is linked to an increase in neurogenesis, an amelioration of memory impairment (Kim et al., 2014; Liu et al., 2011;

Sim, 2014), and enhanced non-amyloidogenic APP processing by activating α-secretase (Nigam et al., 2017).

A large body of literature indicates that a Mediterranean diet consisting of fruits and vegetables, fish, low sugar and fat intake reduces the risk of developing MCI or AD (Morris et al., 2015; Scarmeas et al., 2007). Studies performed worldwide demonstrated that following this type of diet is linked to a lower level of disease-associated risk factors for AD (Gardener et al., 2012; Tangney et al., 2011), showing some degree of protection against episodic memory and cognitive decline in aged population (Loughrey et al., 2017; Trichopoulou et al., 2015; Valls-Pedret et al., 2015). Moreover, Mediterranean diet decreases oxidative stress by reducing intracellular reactive oxidative species (ROS) and apoptosis. In particular, an important intake of vegetables and olive oil is directly linked with longer telomere length and increased telomerase activity (Boccardi et al., 2013; Gu et al., 2015). In particular, the polyphenols included in olives seem to be the active compound that help to prevent AD (Omar et al., 2018), inducing autophagy, decreasing protein aggregation and neuroinflammation, and improving global cognition in AD (Cordero et al., 2018; Grossi et al., 2013).

Two further modifiable factors that negatively affect cognition and motor function are alcohol and smoking. Excessive alcohol drinking causes a deterioration in cognition similar to that found in AD patients (Weissenborn and Duka, 2003). Adolescents with binge ethanol exposure presented loss of cholinergic neurons (Fernandez and Savage, 2017; Vetreno and Crews, 2019) and hippocampal atrophy (Topiwala et al., 2017) like in AD patients. On the other hand, alcoholics such as wine have polyphenols that might have different beneficial effects such as inhibition of Aβ aggregation, reduction of oxidative stress, and inflammation (Dhouafli et al., 2018).

Several studies indicate that smokers have a higher risk to develop dementia and cognitive impairment compared to non-smokers (Anstey et al., 2007; Ott et al., 1998; Reitz et al.,

2007). This increasing risk could be due to the negative effects of smoking on the cardiovascular system, which as mentioned above is another important risk factor of AD (Banks et al., 2019). The negative effects of smoking are confirmed in experimental animals models, in which exposure to cigarette smoke increased tau phosphorylation (Oddo et al., 2005), amyloid deposition and neuroinflammation (Moreno-Gonzalez et al., 2013).

1.3. Preclinical and clinical stage of Alzheimer's disease

AD is a neurodegenerative disorder with the earliest neuropathological changes developing in hippocampus and entorhinal cortex (medial temporal lobe) (Figure 1). These changes induce episodic memory deficits followed by impairment in semantic memory (preclinical AD) (Belleville et al., 2008; Khan et al., 2014; Reitz et al., 2009; Small, 2014). Mild deficits in executive function such as attention, and executive-visuospatial abilities (Bondi et al., 2008) are first observable at the end of the preclinical phase of the disease (Storandt et al., 2006; Twamley et al., 2006). As the AD patient proceeds from the preclinical stage into mild cognitive impairment (MCI), more cognitive functions begin to be impaired including verbal recall (Tuokko et al., 2005). With progression to mild to moderate AD stages, the neuropathology seems to spread further to frontal and parietal lobes, and all cognitive domains become affected. Finally, in severe stages of AD, the neuropathology involves all brain lobes, and impairment is manifesting in all cognitive domains (McKhann et al., 2011).

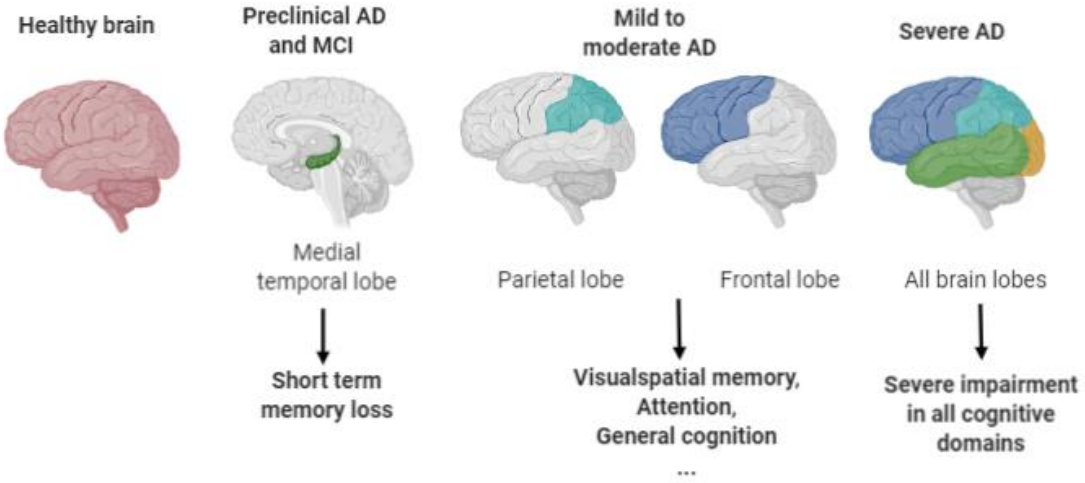

Figure 1: Progression of neuropathology and cognitive impairment in AD.
First, AD neuropathology develops in medial temporal lobe and causes episodic and semantic memory deficits (preclinical AD). As the neurodegeneration spreads to the parietal and frontal lobes, the patient progresses to MCI stage, and higher cognitive domains and general cognition become impaired. In severe stages of AD, neuropathology damages all brain lobes, and profound deficits in all cognitive domains are evident. Graphic was created with BioRender.com.

1.4. Pathophysiological hallmarks of Alzheimer's disease

1.4.1. Brain atrophy

Although a simple visual examination of the brain is not a diagnostic criterion for AD, certain characteristics are highly suggestive of AD neurodegeneration. Using magnetic resonance imaging (MRI), changes in brain volume can be detected very early (Dickerson et al., 2011) and usually an AD brain shows cortical atrophy in primary motor, sensory, and visual cortices and limbic lobe structures (Dickerson et al., 2009). The consequences of this atrophy are an enlargement of sulcal spaces with atrophy of the gyri in the frontal and temporal cortices (Perl, 2010), together with enlargements of the frontal and temporal horns of the lateral ventricles (Apostolova et al., 2012; Serrano-Pozo et al., 2011a) (Figure 2). As a result of this atrophy, brain weight is decreased. Moreover, AD brains usually show a loss of neuromelanin pigmentation in the locus coeruleus (Serrano-Pozo et al., 2011a) (Figure 2). It is important to note that none of these observations are specific for an AD diagnosis.

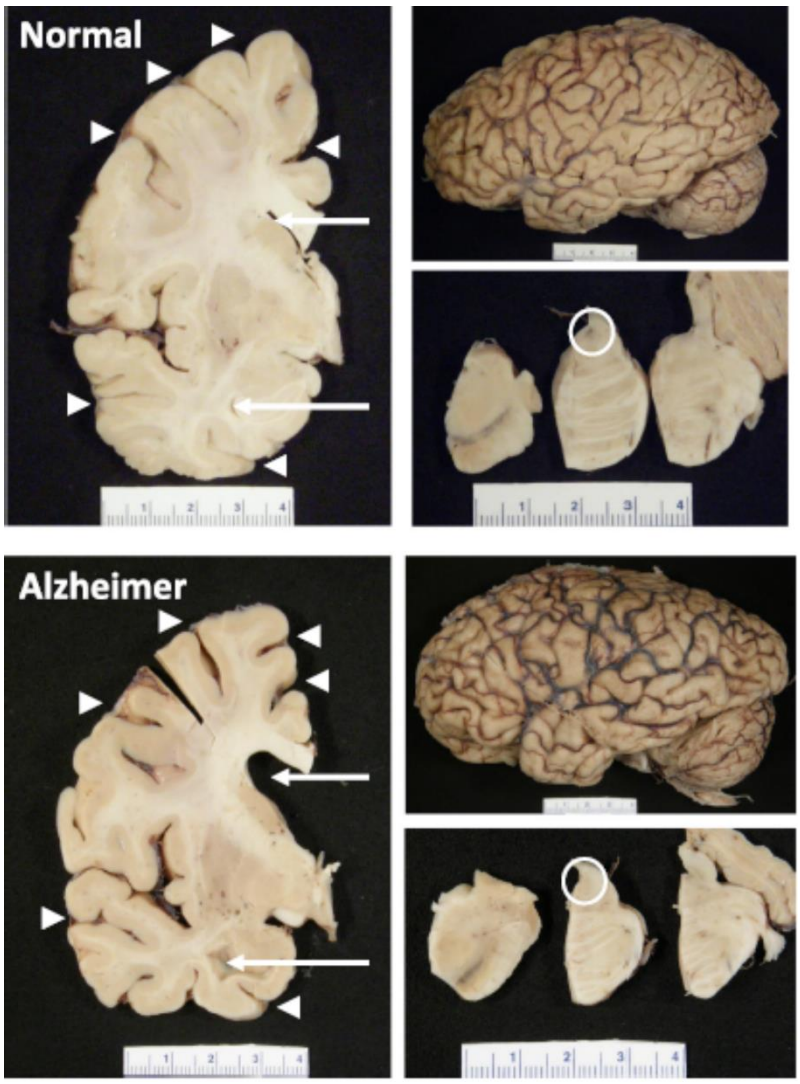

Figure 2: Alzheimer's brain atrophy.
Compared to a normal brain, AD brains present with an enlargement of sulcal spaces and narrowing of gyri (arrowheads). This atrophy is often accompanied by enlargement of the frontal and temporal horns of the lateral ventricles (arrows). Moreover, in the locus coeruleus, a loss of pigmentation in the neurons is frequently observed (open circle). Figure taken with permission from (Deture and Dickson, 2019).

1.4.2. Amyloid deposits

Extracellular amyloid plaques, described for the first time by Alois Alzheimer, represent one of the major hallmarks of AD. They represent extracellular accumulations of $A\beta_{40}$ and $A\beta_{42}$ peptides that result from the aberrant processing of APP by the β- and γ-secretases and an imbalance in production and clearance pathways (Kumar Thakur et al., 2018; Stelzmann et al., 1995; Thal, 2006). A morphological classification, based on staining characteristics, divided the plaques in two main types: diffuse and dense-cored plaques (Dickson, 1997; Thal, 2006). The dense-cored plaques, also called neuritic plaques, present an highly intense

staining to Congo red and fluorescent thioflavin S dyes, implying that they contain more fibrillar forms of Aβ (Davies and Mann, 1993; Thal, 2006; Yamaguchi et al., 1988). Moreover, the dense-cored plaques can be associated with tau-positive dystrophic neurites, synaptic loss, activated microglia and reactive astrocytes (Serrano-Pozo et al., 2011a; Yasuhara et al., 1994). Indeed, the diffuse plaques are amorphous amyloid deposits that are thioflavin S and Congo red negative. They do not show a preferential accumulation of activated microglia and reactive astrocytes and they are also commonly present in the brains of cognitively healthy elderly people (Morris et al., 1996) (Figure 3).

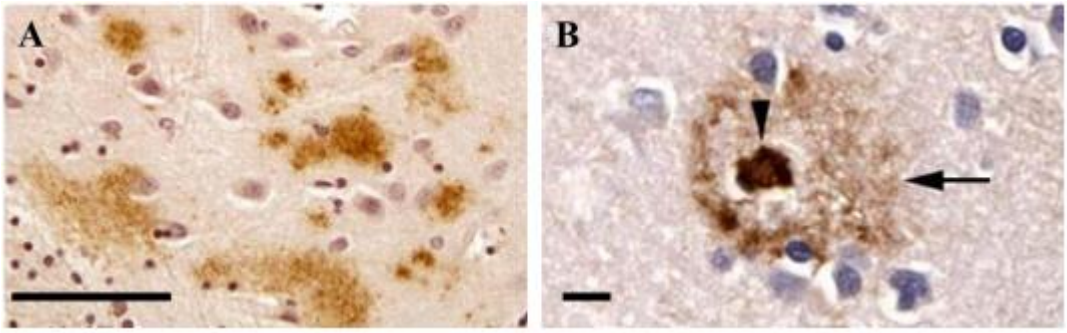

Figure 3: Alzheimer senile plaques.
(A) Diffuse and (B) dense-cored plaques seen by means of immunohistochemistry staining using anti-Aβ antibodies. Scale bar 10μm. Adapted with permission from (Duyckaerts et al., 2009).

1.4.3. Neurofibrillary tangles

The intraneuronal neurofibrillary tangles (NFTs) represent the second neuropathological hallmark of AD and were first described by Alois Alzheimer as filamentous inclusions within the perikaryal area of pyramidal neurons (Vetreno and Crews, 2019). Electron microscopy studies on AD brains showed that NFTs are composed of paired helical filaments (PHFs), having a diameter of 10 nm and forming periodic regular structures of 65–80 nm (Kidd, 1963, 1964; Wiśniewski et al., 1976). Regardless of the constitution, the major component of NFTs was found to be the microtubule-associated protein tau, which is hyperphosphorylated and misfolded. As a consequence, it has been suggested that tau proteins in AD patients have lost their microtubule-stabilizing function of the cytoskeleton

in the axon. This results in an increased aggregation of abnormal tau-protein and a collapse of dendrites and axons of the tangle-bearing neurons (Alonso et al., 1994).

1.4.4. Neuroinflammation

Another neuropathological hallmark of AD is brain inflammation. Under physiological conditions, microglial cells have an important role in the monitoring and the correct turnover of synapses (Wake et al., 2009). In contrast, under stress conditions or in the presence of protein aggregates such as Aβ amyloid fibrils or tau protein filaments, they become activated and are commonly associated with Aβ plaques (Itagaki et al., 1989; Serrano-Pozo et al., 2011b; Vehmas et al., 2003). Moreover, the number of activated microglia cells increases linearly with the neuronal damage due to the NFT burden (Serrano-Pozo et al., 2011b). Together with the activation of the microglia, reactive astrocytes play an important role in the inflammatory response in AD brains (Pike et al., 1995). Normally, astrocytes provide trophic support to neurons and synapses, directly connecting them to the blood-brain barrier (BBB) (Abbott et al., 2006). In AD brains, astrogliosis is mainly observed around senile Aβ plaques, albeit in lower amounts compared to the microgliosis (Steele and Robinson, 2012). In addition, the astrogliosis is observed primarily in later stages of AD and seems to be correlated with tau burden (Vehmas et al., 2003).

1.5. Pathomechanisms of Alzheimer's Disease

1.5.1. The amyloid precursor protein (APP)

The amyloid precursor protein (APP) is a transmembrane protein with a large extracellular N-terminal domain, a transmembrane domain, and a short cytoplasmatic C-terminal domain (Nguyen, 2015; Reinhard et al., 2005) (Figure 4A). In humans, the *APP* gene is located on

chromosome 21 (Dawkins and Small, 2014), and due to this location, several studies correlate the early onset of AD in people with Down syndrome and cognitive impairment to this extra copy of the APP gene (Sosa et al., 2014). Ten different isoforms of APP are known and are generated by alternative splicing of the APP mRNA. The differences between the isoforms are based on the number of amino acids (639–770) (Wang et al., 2017b), and the major ones are APP695, APP751 and APP770 (Chen et al., 2013; Nalivaeva and Turner, 2013). These different isoforms of APP are differentially expressed in cell types and tissues. While APP695 is predominantly expressed in neurons, APP751 and APP770 are more commonly expressed in peripheral tissues (Mattson, 1997). APP is synthesized in the endoplasmic reticulum (ER) and is transported to the cell membrane through the Golgi networks (Greenfield et al., 1999; Hartmann et al., 1997; Xu et al., 1997). Before being integrated in the plasma membrane, the immature APP undergoes post-translational modifications such as N- and O-glycosylations, phosphorylations, sulfations in tyrosine residues and palmitoylations (Bhattacharyya et al., 2013; Buoso et al., 2010; Wang et al., 2017b). Under physiological conditions, APP positively modulates cells growth and survival (Thinakaran and Koo, 2008) and promotes neuritic arborisation (Leyssen et al., 2005). It further plays important roles in the generation and maintenance of dendritic spines (Lee et al., 2010), as well as in regulating neural activity, plasticity and memory (Roch et al., 1994; Turner et al., 2003).

1.5.2. Amyloidogenic and non-amyloidogenic processing of APP

Mature APP can be processed by two enzymatic pathways, namely the canonical non-amyloidogenic and the non-canonical amyloidogenic pathway, depending on secretase specific cleavage sites. The non-amyloidogenic pathway is localized mostly in the cell membrane (Lammich et al., 1999) and is predominant in physiological conditions

(Agostinho et al., 2015). Briefly, in the non-amyloidogenic pathway (see Figure 4B) APP is cleaved first by the α-secretase within the Aβ domain, resulting in the production of a soluble fragment (sAPPα) that it is released to the extracellular space, and the α C-terminal fragment (αCTF), known as C83, in the cell membrane. Next, the C83 fragment is cleaved by the γ-secretase leading to the release of the soluble peptide P3 to the extracellular medium and the APP intracellular domain (AICD) to the cytoplasm (Grimm et al., 2013; Haass et al., 2012). As APP is cleaved within the Aβ domain, the generation and aggregation of Aβ is thereby prevented (Haass et al., 2012) (Figure 4B).

In the amyloidogenic pathway, however (Figure 4C), β-secretase cleaves APP outside the Aβ domain, leading to the generation of Aβ peptides. APP is first cut by β-secretase, producing the soluble APPs-β domain that is released to the extracellular medium while the remaining β-C-terminal fragment (β-CTF), known also as C99, is retained in the cell membrane. Next, the C99 fragment is further cleaved by γ-secretase generating and releasing Aβ peptides to the extracellular medium and the AICD fragment to the cytoplasm (Grimm et al., 2013; Haass et al., 2012). Due to the presence of multiple cleavage sites for γ-secretase, Aβ peptides of various length range (37 to 43 amino acids) are produced (Marcello et al., 2011). The most common isoform under physiological conditions is $A\beta_{1-40}$ (Citron et al., 1995; Haass et al., 1992), while the longer form $A\beta_{1-42}$ is more prone to form toxic oligomers (Thinakaran and Koo, 2008).

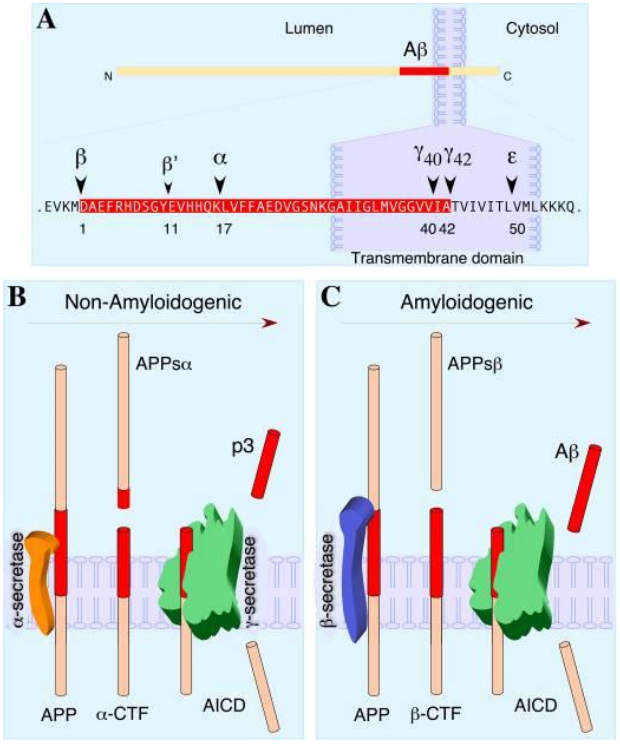

Figure 4: Schematic diagram of APP proteolytic processing.
(A) Schematic representation of APP, with the Aβ domain coloured in red and enlarged. The principal cleavage sites of α, β- and γ-secretases are marked along with Aβ amino acids numbering from the N terminus of Aβ (Asp1). (B) non-amyloidogenic and (C) amyloidogenic processing of APP. Figure taken with permission from (Thinakaran and Koo, 2008).

1.5.3. The amyloid cascade hypothesis

The amyloid cascade hypothesis has been the principal explanation for the pathomechanism of AD for over 25 years. According to this hypothesis, Aβ deposition and aggregation is the main origin of the neurodegeneration in AD (Hardy and Allsop, 1991; Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Selkoe, 1991). This hypothesis is based on two main key observations: (i) the finding that Aβ plaques are mainly constituted of Aβ proteins (Glenner and Wong, 1984) and (ii), the discovery that AD could be inherited in an autosomal dominant form (Goate et al., 1991). The first mutation identified was in the *APP* gene (Goate et al., 1991). As already explained in detail in section 1.5.2, APP is the protein from which Aβ peptides are generated after β- (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) and γ-secretase cleavage (De Strooper et al., 1998; Wolfe et al., 1999). This hypothesis was further supported by the identification of further mutations in the presenilin 1 and 2 (*PSEN1/2*) genes (Levy-Lahad et al., 1995; Rogaev et

al., 1995; Sherrington et al., 1995) in the EO-FAD form of the disease. These two proteins are homologous and can form the catalytic active site of the γ-secretase complex (Edbauer et al., 2003). These mutations are responsible for the alteration of the proteolytic cleavage of APP, causing an increased ratio between longer and more aggregating $A\beta_{42}$ species respect the shorter $A\beta_{40}$ forms (Hardy, 1997). Furthermore, another observation supporting the amyloid hypothesis is that patients with trisomy 21 (Down syndrome) manifest an AD-like pathology very early in life, starting at around an age of 40 years (Kolata, 1985). This is been attributed to the triplication of the gene coding for *APP*, which is located on the chromosome 21. It was reported that in the brain of Down's syndrome patients, the amount of both APP and Aβ was increased compared to healthy individuals (Kolata, 1985), and that this *APP* locus duplication might cause a cerebral amyloid angiopathy syndrome (Delabar et al., 1987; Rovelet-Lecrux et al., 2006). During the years, the amyloid cascade hypothesis has been supported by the generation of genetically modified APP or APP/PS1 mouse models that have been shown to mimic some of the main histopathological and behavioural hallmarks of EO-FAD (Puzzo et al., 2014). On the other hand, many of these mouse models only mimic the Aβ accumulation and the formation of senile plaques, without taking the other factors associated to AD, such as NFT formation, the nerve cell death (Arendash and King, 2002; Hsiao et al., 1996), and memory deficits like in the Tg2576 and PS1+APP mice into consideration (Arendash and King, 2002; Holcomb et al., 1999; Kobayashi and Chen, 2005; Spires and Hyman, 2005). As an example, the $A\beta_{42}$-overexpressing BRI2-Aβ mice, which had clear Aβ deposits and plaques in the brain, do not present neuronal loss or/and cognitive impairments (Kim et al., 2007, 2013). Taken together, these observations suggest that the Aβ peptides are not cytotoxic *per se* and that there is no clear correlation between Aβ formation and subsequent induction of tau accumulation. In addition to these pre-clinical evidences, different therapies that have Aβ as a target effectively decreased brain Aβ accumulation but fail to impact on cognitive deficits or the accumulation of tau (Doody et

al., 2014; Giacobini and Gold, 2013; Ostrowitzki et al., 2012; Salloway et al., 2014). Interestingly, sometimes the brains of elderly non-AD patients had a plaque amount and distribution similar to AD patients (Chételat et al., 2013; Davis et al., 1999; Fagan et al., 2009; Price et al., 2009), suggesting that Aβ amyloid formation has no direct correlation with AD onset and is a natural phenomenon of aging. Taking all these pieces of evidence together, it is also possible that, discordant to the amyloid hypothesis, amyloid accumulation, neurodegeneration, neuronal loss, and cognitive impairment are independent, non-related phenomena (Chételat, 2013).

Over the years, the amyloid hypothesis has been modified. A revised version focused the attention on the crucial role of intraneuronal Aβ accumulation before the deposition of extracellular plaques (Wirths et al., 2004). In 1985, the first evidence of intracellular deposition of Aβ within neurons was reported by Masters et al. (Masters et al., 1985a). Different immunohistochemical studies have shown that the intraneuronal Aβ staining was most prominent in pyramidal neurons of entorhinal cortex and hippocampus, which are the brains regions with beginning neurodegeneration (Gouras et al., 2000). Interestingly, analysis of *post-mortem* AD brains, revealed intracellular Aβ accumulation before the formation of paired helical filaments (PHF), indicating that intra-cellular deposits are among the first and crucial changes in AD brain (Fernández-Vizarra et al., 2004). Moreover, the same immunohistochemical pattern was found in young Down syndrome patients, with the intraneuronal $A\beta_{42}$ accumulation preceding plaque formation (Gyure et al., 2001; Mori et al., 2002). Further evidence for the influence of intracellular Aβ was provided by studies in mouse models of AD. In triple-transgenic mice (3xTg-AD), intraneuronal Aβ accumulation correlates with LTP and cognitive deficits and these events appeared prior to plaque and NFT deposition (Billings et al., 2005; Oddo et al., 2003). Moreover, as already mentioned before, most of the classical AD mouse models presented some of the hallmarks of AD such as the plaque accumulation or neurofibrillary tangle deposition, however without showing

brain atrophy and neuronal loss (Wirths et al., 2004). More thorough analyses of models such as Tg2576 (Hsiao et al., 1996), APP/PS1KI (Blanchard et al., 2003; Casas et al., 2004; Schmitz et al., 2004; Wirths et al., 2006), 5xFAD (Eimer and Vassar, 2013; Jawhar et al., 2012; Oakley et al., 2006), and Tg4-42 (Bouter et al., 2013) revealed coincidence of early Aβ accumulation with neuronal loss and cognitive deficits (Wirths and Zampar, 2020). Thus, present data suggests that both extra- and intra-cellular Aβ are important players in the development of AD pathogenesis.

1.5.4. The cholinergic hypothesis

The cholinergic hypothesis originates from three main discoveries made in the 1970s. First, the involvement of the cholinergic system in AD was postulated by Peter Davies and A. J. F. Maloney in 1976 (Davies and Maloney, 1976). They analysed the activities of some essential enzymes implicated in the synthesis of neurotransmitters in twenty regions of control and AD brains. They discovered that both the activity of choline acetyltransferase (ChAT) and the concentration of acetylcholine (ACh) were significantly reduced in the amygdala, hippocampus, and cortex in AD brains,(Bowen et al., 1976; Fotiou et al., 2015; Francis et al., 1999; H. Ferreira-Vieira et al., 2016; White et al., 1977). ChAT is the key enzyme for the synthesized ACh using choline and acetylcoenzyme A as precursors. Secondly, late stage AD is characterized by a drastic loss of neurons from the nucleus basalis of Meynert (NBM) in the forebrain, the main source of cortical cholinergic neuronal fibres (Mesulam, 2013; Whitehouse et al., 1981).

The idea of a crucial role of the cholinergic system in AD was additionally reinforced by studies in humans showing that low doses of scopolamine, a muscarinic antagonist, caused cognitive deficits in young subjects. These deficits were similar to the ones already observed in aged participants (Drachman and Leavitt, 1974).

Therefore, as a logical consequence, several drugs stimulating the cholinergic system started to be used in pre-clinical and clinical studies. The best results were achieved from physostigmine, an anticholinesterase drug, which showed beneficial effects on cognition in monkeys (Bartus, 1979), young and elderly humans (Drachman, 1980), and AD patients (Muramoto et al., 1979). A more thorough description of the pharmacological treatment related to the cholinergic hypothesis will be done in the section 1.6.1.1.

1.5.5. The glutamate hypothesis

Glutamate is a non-essential amino acid that it is not able to cross the blood-brain barrier (BBB). It is synthesized in the central nervous system (CNS) from glutamine by the enzyme glutamine synthetase. Glutamate is the principal excitatory neurotransmitter in the CNS (Danysz and Parsons, 2012; Frisardi et al., 2011; Gao and Bao, 2011; Revett et al., 2013), and it is involved in neuronal growth and differentiation, synaptic transmission and plasticity, as well as learning and memory process (Butterfield and Pocernich, 2003; Francis, 2003). The glutamatergic neurons are located exactly in areas that are affected in AD, such as the hippocampus (Francis, 2003) and the pyramidal neurons in layers III and V of the neo-cortex (Bussière et al., 2003; Kowall and Beal, 1991). One of the essential functions of the glutamatergic system is to control glutamate concentration in the synapse cleft. In pre-synaptic neurons, the vesicular glutamate transporters (VGLUT) keep the glutamate stored in vesicles (Fremeau et al., 2001), and only upon plasma membrane depolarization it becomes released into the synaptic cleft, where it binds to its receptors (Danbolt, 2001; Kew and Kemp, 2005; Ozawa et al., 1998).

Glutamate receptors are divided into two different classes: ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors. The first are further classified into three sub-types, which differ according to their specific agonist or cations' permeability: N-methyl-D-

aspartic acid (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA) receptors; the NMDA receptors are principally Ca_2^+ ion permeable, while the AMPA and KA are mainly permeable to Na^+ and K^+ ions (Kew and Kemp, 2005).

Regarding the mGluR, eight different G-protein coupled receptors can be distinguished (Kew and Kemp, 2005). Moreover, astroglial cells express the so-called excitatory amino acid transporter (EAAT), that is essential for the re-uptake of glutamate from the synaptic cleft (Kim et al., 2011).

The glutamatergic hypothesis of AD, is based on different studies that reported reduced levels of VGLUT receptors (Kashani et al., 2008; Kirvell et al., 2006; Sokolow et al., 2012) and EEAT transporters in prefrontal or parietal cortex and hippocampus of AD patients when compared with healthy controls (Jacob et al., 2007; Li et al., 1997; Masliah et al., 1996). These findings suggested an attenuation of glutamate clearance from the synaptic cleft and claimed that changes in the expression levels of glutamate transporters are early events in the AD neurodegeneration process (Masliah et al., 1996). Together with the reduction of EAAT transporters, a decrease in glutamine synthetase enzyme levels was also reported (Robinson, 2001). Moreover, neurodegeneration also seems to be related to an Aβ interaction with the different receptors and transporters involved in the glutamate metabolism (Esposito et al., 2013). Taken together, these changes lead to an increased glutamate concentration at the synaptic cleft, which activates NMDA receptors and the subsequently increases calcium influx (Dong et al., 2009; Greenamyre et al., 1988). This might trigger neuronal cell death through the activation of signalling pathways (Alberdi et al., 2010; Dong et al., 2009; Vosler et al., 2008), correlating with cognitive decline in AD patients (Wenk, 2006; Wenk et al., 2006). For details see Figure 5.

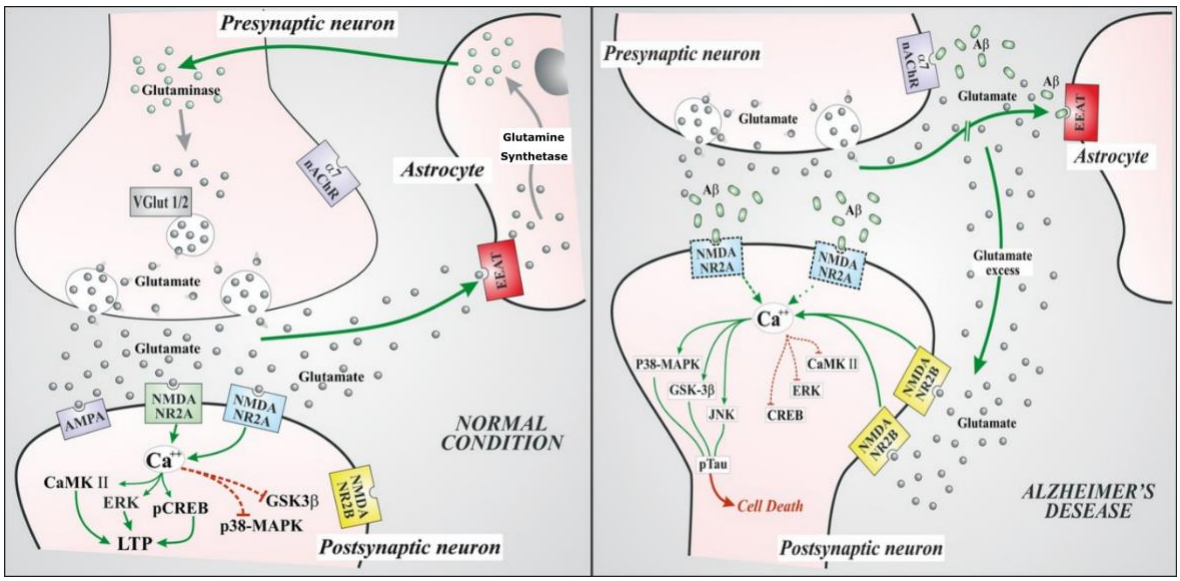

Figure 5: Physiological and AD glutamate-mediated transmission at the synaptic cleft.
Glutamate released from presynaptic neurons binds its ionotropic receptor on the post-synaptic neuron. In the present figure the attention is focused on the role of the NMDA receptor signalling both in physiological conditions, as well as AD. Under normal conditions, the activation of NMDA NR2A receptor promotes an increase in intracellular calcium, which activates (CaMK, ERK, and CREB) or inhibits certain signalling pathways (GSK3β and MAPK). The first one promotes long-term potentiation (LTP) mechanisms while the second is involved in long-term depression (LTD) and synaptic remodelling. Glutamate, via EAAT transporters, is re-uptaken in astrocytes, where it is converted to glutamine which returns to the pre-synaptic neuron. Here, the glutamine it is transformed again in glutamate via the glutaminase enzyme and loaded in vesicles through VGlut transporters. In AD, Aβ interferes with different glutamate receptors and transporters causing an overall high glutamate concentration in the synaptic cleft. Therefore, increased calcium influx causes the activation of pathological pathways, which are responsible for neuronal loss. VGlut (vesicular glutamate transporter); EAAT (excitatory amino acid transporter); a7-nAchR (alpha-7 nicotinic acetylcholine receptor); NMDANR2A (N-methyl-D-aspartate NR2A subunit); NMDANR2B (N-methyl-D-aspartate NR2B subunit); ERK (extracellular signal-related kinase); CaMKII(calcium calmodulin-dependent kinase II); CREB (cyclic AMP response element binding protein); pCREB (phosphorylated cyclicAMP response element binding protein); GSK3b (glycogen synthasekinase 3b); p38-MAPK (p38 mitogen-activated protein kinase) JNK (c-Jun N-terminal-kinase), pTau (hyper-phosphorylated tau protein). Figure adapted with permission from (Esposito et al., 2013).

1.6. Therapeutic strategies

Currently, there are only four drugs approved by the Food & Drug Administration (FDA) divided in two categories: acetylcholinesterase inhibitors (AChEIs; donepezil, rivastigmine, and galantamine) and an antagonist of the N-methyl-D-aspartic acid (NMDA) receptor (memantine). These drugs are neurotransmitter regulators, and they can only relieve the symptoms or delay AD progression. Most of the current trials failed in phase 3 (Long and Holtzman, 2019), mainly because they intervened on patients with mild-to-moderate symptomatic AD, and despite this stage is considering being an intermediate phase, in terms of neurodegeneration it is quite advanced. At this disease stage, an irreversible loss of neurons and synapses can be already observed in the brain (Gómez-Isla et al., 1996; Vermunt

et al., 2019). Currently there are more than 100 different compounds tested in different phases of clinical trials (Hara et al., 2019). Due to the ineffectiveness of the few approved therapies for the treatment of AD, enormous efforts have been made to discover new molecules with the potential to halt the progression of neurodegeneration, the so called "disease-modifying therapies".

1.6.1. Symptomatic treatments of Alzheimer's disease

1.6.1.1. Acetylcholinesterase inhibitors

Acetyl-cholinesterase inhibitors (AChEIs) can mitigate the cognitive deficits observed in AD patients by preventing the degradation of acetylcholine (Anand and Singh, 2013; Deardorff et al., 2015; Håkansson, 1993; O'Regan et al., 2015). In 1995, the FDA approved tacrine, the first AChEIs (Davis and Powchick, 1995), but it was discontinued due to its liver toxicity (Blackard et al., 1998; Watkins et al., 1994). At present, second generation inhibitors are used including donepezil, rivastigmine, and galantamine (Bullock et al., 2005; Wilcock et al., 2003).

Donepezil was approved in 1996 for the treatment of mild-to-moderate AD with doses of 5 or 10 mg/day, and later in 2006 for severe AD (Farlow et al., 2010; Rogers and Friedhoff, 1996). Rivastigmine was approved for the treatment of mild-to-moderate AD by FDA in 2000, and is a "pseudo-irreversible" inhibitor (Noetzli and Eap, 2013). It is administered in capsule form, oral solution or as transdermal patch at dosages between 6-12 mg/day (Boada and Arranz, 2013; Small and Dubois, 2007). First approved in 2001 galantamine, a reversible competitive inhibitor of acetylcholinesterase and allosteric modulator of nicotinic acetylcholine receptors, is indicated for treatment in patients with mild-to-moderate AD. It is administrated in tablets or oral solution with a dosage in a range of 16–24 mg/day (Lilienfeld, 2002). In comparison with the first inhibitor, these drugs shown less side effects

and better improvement in general cognition in patients with mild to moderate AD. Patients treated with rivastigmine and galantamine had better daily living abilities than those treated with donepezil (Bullock et al., 2005; Mintzer and Kershaw, 2003; Wilcock et al., 2003).

1.6.1.2. Memantine

Amyloid-β peptides are able to bind different types of receptors, among them are glutamatergic NMDA receptors (Danysz and Parsons, 2012). This binding causes an increase of the cytosolic calcium concentration (Macdermott et al., 1986; Texidó et al., 2011), and results in mitochondrial dysfunction, an increment of ROS production and oxidative stress, and finally cell death (Peng and Jou, 2010). Therefore, the use of NMDA receptor antagonists is suggested as a promising therapeutic target in AD. In this regard, memantine (3, 5-dimethyldamantane-1-amine) is the most prominent and widely studied molecule of this class. Memantine, an uncompetitive NMDAR antagonist, was approved by FDA in 2003 for the treatment of moderate-to-severe AD (Lo and Grossberg, 2011). This drug exhibits its inhibitory activity only when the receptor channel is in an open state. Memantine enters and binds the receptor, thereby blocking the cation pore and preventing the ion flux. It leaves the receptor in a depolarized condition so that synaptic activity is maintained (Rogawski and Wenk, 2003). The efficacy and safety of the drug has been assessed in a variety of studies using AD mouse models. Minkeviciene and colleagues observed that oral administration of 30 mg/kg/day of memantine for one month completely rescued spatial memory learning deficits in the Morris water maze (MWM) task in APP/PS1 mice (Minkeviciene et al., 2004). Another study, using heterozygous APP23 mice, reported the same finding with drug-treated animals showing improved abilities in the MWM memory task (Van Dam and De Deyn, 2006). Moreover, treatment with memantine in 3x-TgAD mice not only improved mice performance in the MWM but also in the novel object

recognition task (NOR) and in the passive avoidance test, demonstrating the effect of memantine on different types of memory (Martinez-Coria et al., 2010). Since memantine was reported to be effective and safe in pre-clinical *in vivo* study, a series of clinical trials in human patients was carried out. In general, results from these trials revealed that memantine-treated patients had a three times higher probability to preserve their life independency (Rive et al., 2004) together significantly slower cognitive decline after 24 or 28 weeks of treatment (Peskind et al., 2006; Reisberg et al., 2006). Moreover, memantine also moderately improved the language impairment observed in AD patients (Ferris et al., 2009).

1.6.2. Disease-modifying treatments

1.6.2.1. Aβ-directed therapeutics

According to the amyloid hypothesis, Aβ accumulation is a key factor in AD pathogenesis. Therefore, the aim of most of Aβ targeting drugs is to decrease Aβ levels and deposition in the brain and three main strategies were developed to target Aβ in clinical trials: (i) secretase inhibitors and (ii) active or (iii) passive immunization.

The first strategy is to reduce Aβ production by the inhibiting β- and γ-secretase activities. Peters et al. have shown that β-secretase inhibition limits the initial process of Aβ plaque accumulation without having an effect on prevention on plaque growth in the APP/PS1 mouse model of AD (Peters et al., 2018). This finding indicates that β-secretase inhibition could be effective as a prophylactic strategy. During the last years, different types of β-secretase inhibitors entered clinical trials. Two of them, LY3202626 and LY2886721, failed in phase II due to deteriorated cognitive performance and liver toxicity, respectively. Five additional β-site cleaving enzyme 1 (BACE1) inhibitors (Verubecestat, Lanabacestat, Atabecestat, Umibecestat, and Elenbecestat) have recently failed in phase III clinical trials due to a worsening in cognitive performance and accompanying side effects like weight loss

(Egan et al., 2018, 2019; Henley et al., 2019; Lopez Lopez et al., 2019). Currently, no further β-secretase inhibitors are investigated in clinical trials.

Several γ-secretase inhibitors have been evaluated in clinical trials. Avagacestat reached phase II but was discontinued because of a lack of efficacy to ameliorate cognitive decline and several side effects such as nausea, diarrhoea and an increased risk to develop skin cancer (Coric et al., 2015). Semagacestat was the first γ-secretase inhibitor entering a phase 3 trial for AD, however, the trial was interrupted early due to an increased incidence of skin cancers and infection, weight loss and worsened cognition (Doody et al., 2013).

Active immunization therapy in AD has been studied for the last 20 years (Gallardo and Holtzman, 2017). In 1999, Schenk et al. for the first time demonstrated successful proof-of concept data of an antibody-mediated therapy. They actively immunized PDAPP mice using full-length human $A\beta_{42}$ peptide and observed a significant reduction in β-amyloid plaque load in aged, together with a prevention of formation of new deposits in young vaccinated animals (Schenk et al., 1999). Other studies reported related results together with an amelioration in cognitive deficits using different AD mouse models (Dodart et al., 2002; Janus et al., 2000).

Given these promising pre-clinical results, a series of human clinical trials was approved. The initial AN1792 trial had to be discontinued in phase II due to the development of aseptic meningoencephalitis. Furthermore, *post-mortem* analysis of brains from immunized patients revealed a significant variability of the efficacy of Aβ clearance (Nicoll et al., 2019; Vellas et al., 2009). Active vaccination therapies are still being investigated, with ABVac40 being currently evaluated in a phase II clinical trial. It is suggested that the risk of developing meningoencephalitis is avoided by removing parts of the Aβ peptide responsible for triggering an immune responses (Lacosta et al., 2018).

Passive immunization is another approach for immunotherapy targeting Aβ. The main advantage of this strategy is a reduced risk of adverse events because the titre of the

monoclonal antibody are strictly controlled (Lannfelt et al., 2014). Bapineuzumab, was the first and most extensively antibody studied in AD patients. It is the humanized form of the mouse 3D6 antibody that successfully reduced plaque in the PDAPP mouse model of AD (Bard et al., 2000). The antibody targets aggregated fibrillary Aβ and binds the N-terminus of the peptide ($A\beta_{1-5}$) (Miles et al., 2013). Phase III trials in mild-to-moderate AD did not report any effect of an amelioration of cognition or activities of daily living (Salloway et al., 2014) and more importantly, brain imaging abnormalities with oedema and effusion have been observed (Liu et al., 2018).

Other humanized anti-Aβ antibodies such as solanezumab (Doody et al., 2014; Honig et al., 2018), crenezumab (Cummings et al., 2018), and gantenerumab (Bohrmann et al., 2012; Ostrowitzki et al., 2012) were discontinued for the same reasons of bapinezumab (Loureiro et al., 2020).

1.6.2.2. Tau-directed therapeutics

Numerous strategies have been postulated to reduce AD tau pathology. The first compound investigated in clinical trial was methylene blue, which decreased tau aggregation, induced autophagy and attenuated tauopathy in mouse models (Congdon et al., 2012; Crowe et al., 2013; Wischik et al., 1996). Unfortunately, when used in clinical trials, there was no amelioration in cognition so it was discontinued in phase III (Gauthier et al., 2016). The second strategy for reducing tau pathology is to inhibit its hyperphosphorylation. Tideglusib, a glycogen synthase kinase (GSK) 3 inhibitor, was tested *in vivo* using transgenic APP^{sw}-tau^{vlw} mice. It was reported that inhibition of GSK3 was able to decrease tau phosphorylation together with a reduction of Aβ accumulation and rescue neuronal loss (Serenó et al., 2009). However, a phase II clinical trial with tideglusib did not ameliorate cognitive deficit in mild-to-moderate AD patients (Lovestone et al., 2015).

As for Aβ, companies started to develop immunotherapies including both active and passive immunization to promote tau clearance from AD brain. Regarding the active immunization, two compounds are being investigated. The first one is AADvac-1 that completed phase I and currently is in phase II trials (Novak et al., 2019) and ACI-35 which have the aim to create an immune response only against pathologic hyperphosphorylated tau (Theunis et al., 2013).

Various tau-directed monoclonal antibodies are currently studied in clinical trials. The humanized version of mouse anti-human tau monoclonal antibody HJ8.5 (Yanamandra et al., 2013, 2015) has been tested in a phase II trial including patients with early AD (West et al., 2017). A different Biogen humanized anti-tau monoclonal antibody, BIIB092, has been investigated in a phase II study and was shown to reduce N-terminal tau in CSF of patients (Boxer et al., 2019).

1.6.3. Alternative therapeutic approaches

As already mentioned in section 1.2.2, recent prospective studies have shown that certain lifestyle factors are associated with increased risk of developing AD, suggesting that primary intervention may be an alternative and applicable strategy to reduce the incidence of AD (Bendlin et al., 2010; Norton et al., 2013). Moreover, as already discussed in section 1.6.1, the only currently drugs approved for AD have poor efficacy and do not slow the neurodegeneration progression, which might be partially due to a late administration when neurons and synapses are irremediably lost. To decelerate or even stop AD progression, it seems crucial to target neurological factors that are compromised at an early stage of the disease.

1.6.3.1. Caffeine

Caffeine, a central nervous system stimulant, is the world's most consumed psychoactive drug (Nehlig et al., 1992). It has been assessed that in 2019-2020,~ 167 million of 60 kilogram bags of coffee (International Coffee Organization, 2020) are consumed worldwide, with an average coffee consumption in the range from 5,4 (Italy and France) to 11,7 (Finland) kg/year per capita in European countries (Barcelos et al., 2020). Caffeine, an adenosine receptor antagonist, is rapidly and completely absorbed via the gastrointestinal tract (Newton et al., 1981), and due to his structure similarity to adenosine, can easily cross the BBB and reach the brain. Caffeine is primarily metabolized in the liver by the cytochrome P450 oxidase system to paraxanthine, theophylline and theobromine (Newton et al., 1981; Roberts et al., 1994). As a brain stimulant, the principle effects of caffeine are to enhance alertness, ameliorate the ability to sustain intellectual activity, decrease reaction times and improve cognition and learning (Franke and Lieb, 2010). Currently, epidemiological evidence suggests that coffee intake may be inversely related with different diseases and conditions, such as type 2 diabetes (Ding et al., 2014), cardiovascular (Ding et al., 2015) and neurodegenerative diseases (Madeira et al., 2017). Moreover, several human studies suggested that a daily consumption of caffeine, equivalent to 3 or more cups of coffee, reduces cognitive decline in women and men without dementia (van Gelder et al., 2007; Ritchie et al., 2007). Other studies focused on the possible positive effects of caffeine in AD patients. One of them, a 21-years follow-up study, reported that starting to ingest 3-5 cups of coffee per day at midlife reduced the risk of AD in later life (Eskelinen et al., 2009). Interestingly, another study showed that AD patients consumed considerably less caffeine during the 20 years preceding AD diagnosis when compared to age-matched no-AD patients (Maia and De Mendonça, 2002). These human studies propose that long-term caffeine consumption might be protective against AD.

1.6.3.2. Riluzole

Riluzole (2-amino-6-(trifluoromethoxy) benzothiazole), a glutamate release modulator (Cheah et al., 2010), represents the only FDA-approved drug for the treatment of amyotrophic lateral sclerosis (ALS) (Bellingham, 2011; Cheah et al., 2010). Although the mechanism of action of riluzole is not fully understood, it seems to play an important role in the inhibition of sodiumchannels, with the consequence of decreased presynaptic glutamate release and a facilitation of glial extracellular glutamate re-uptake by increasing EAAT2 gene expression (Azbill et al., 2000; Diao et al., 2013; Fumagalli et al., 2008; Pereira et al., 2017; Dos Santos Frizzo et al., 2004). Preliminary human and animal studies suggest an alternative use of this compound for the treatment of other neurodegenerative diseases such as Parkinson's disease and AD (Carbone et al., 2012; Hunsberger et al., 2015; Pittenger et al., 2008; Zarate and Manji, 2008). Preclinical *in vivo* evidence suggested riluzole as a new potential therapeutic drug to prevent age-related cognitive decline in AD (Brothers et al., 2013; Hunsberger et al., 2015; Pereira et al., 2014). It has been shown that riluzole administration induced the clustering of dendritic spines in the hippocampus (Kavalali et al., 1999; Kleindienst et al., 2011; Pereira et al., 2014). Clustering is a critical process of synaptic plasticity and is essential to increase synaptic strength (Trachtenberg et al., 2002). This suggests that riluzoles potential efficacy is directly linked to its role as modulator of synaptic plasticity (Larkum and Nevian, 2008; Polsky et al., 2004). Additionally, riluzole rescues age-related gene expression changes in the hippocampus and alters gene signalling pathways related to AD pathology (Okamoto et al., 2018; Pereira et al., 2017). These preliminary data demonstrate that riluzole could have a disease modifying effect in preclinical models of AD and might be used as an alternative glutamate modulator in an early phase of the disease.

1.6.3.3. The cognitive reserve capacity hypothesis and environmental interventions

In 1988, for the first time, Mortimer hypothesized a link between poor education and risk of developing dementia (Mortimer, 1988). Since that, different epidemiological studies examining this hypothesis all around the world were carried out (Friedland, 1994; Katzman, 1993; Mortimer, 1997), with the majority of them finding that insufficient education represents an important risk factor for developing AD (Delabar et al., 1987; Friedland, 1993; Stern et al., 1994; White et al., 1994; Zhang et al., 1990). Not only inadequate education contributes to an increased AD risk, but also other lifestyle factors such as a lack of social interaction, physical exercise, and leisure activity (Arenaza-Urquijo et al., 2015; Fratiglioni et al., 2004; Livingston et al., 2017; Nithianantharajah and Hannan, 2011; Pope et al., 2003; Scarmeas and Stern, 2003).

These observations promote the hypothesis of a brain cognitive reserve (CR) capacity, offering a potential preventive strategy against dementia (Stern, 2006, 2012). This concept hypothesized that people having more mental activity in the entire life develop stronger neuronal networks, that can more efficiently tolerate pathological brain damage (Stern, 2006, 2012). Therefore, CR links neuroplasticity to cognitive experience, and in this way the brain might have a reserve for its functional maintenance despite possible damage (Arenaza-Urquijo et al., 2015; Clare et al., 2017; Wang et al., 2017a).

Cerebral aging is defined by relevant inter-person differences (Raz et al., 2005). This does not only rely on structural, metabolic or chemical alterations in the brain (Bäckman et al., 2010; Cabeza, 2002; Grady et al., 2006), but also in the compensation capacity to deal with the brain changes due to natural aging (Cabeza et al., 2002) or the development of a neurodegenerative disease (Grady et al., 1995). In fact, numerous studies point out that pathological alterations in AD brain do not always correlate with clinical symptoms (Fratiglioni et al., 1991; Katzman et al., 1988; Ott et al., 1999; Schmand et al., 1997) and

that a comparable severity of brain damage induces various levels of cognitive deficits among individuals (Kesler et al., 2003).

The cognitive-reserve hypothesis is well supported by a variety of studies investigating environmental manipulation in rodents. The environmental enrichment (EE) paradigm is an experimental setting, used to promote cognitive stimulation. Rodents are housed in a stimulating environment that enhances social interaction and sensory, cognitive and motor activities compared to standard housing conditions (Nithianantharajah and Hannan, 2006). Usually, enriched cages are compared to standard cages used regularly in animal facilities and are equipped with different type of colourful objects, running wheels, climbing items, houses, tunnels and nesting materials. The regular re-arrangement of the environment ensures new sensory-motor and cognitive stimuli, thereby stimulating learning and memory skills (Nithianantharajah and Hannan, 2006; van Praag et al., 2000).

The EE paradigm has been used in several AD mouse models, mostly resulting in an amelioration of AD pathology. Liu et al. demonstrated that in APP/PS1 mice, five months of long-term treadmill activity improved not only cognitive deficits and LTP but also reduced Aβ deposition and tau phosphorylation (Liu et al., 2013). Other studies reported related results with regard to Aβ accumulation in other rodent models of AD such as Tg2576 (Nichol et al., 2008), 3xTg-AD (García-Mesa et al., 2012), and TgCRND8 (Adlard et al., 2005).

Other key characteristics of AD-related alterations such as impaired neurogenesis, synaptic and neuron loss seem to be also positively influenced by EE housing. In the Tg4-42 mouse model of AD, it was reported that EE housing or physical activity decreased the amount of hippocampal neuron loss (Hüttenrauch et al., 2016a) and ameliorated impaired hippocampal neurogenesis (Gerberding et al., 2019; Hüttenrauch et al., 2016a).

Rodents housed in EE display significant changes in neuronal connectivity, synaptic morphology and in general brain biochemical parameters when compared with animals

housed under standard conditions (van Praag et al., 2000). These neuroanatomical changes correlate well with improved performance in behavioural tasks such as contextual fear conditioning (Duffy et al., 2001; Rampon et al., 2000; Woodcock and Richardson, 2000), two-way active avoidance (Escorihuela et al., 1994), delayed alternation (Winocur and Greenwood, 1999) or the MWM task (Park et al., 1992; Saari et al., 1990; Wainwright et al., 1993).

Despite all these promising results, there is still some debate regarding the efficacy of EE on the clearance of Aβ deposits, and this could be due to the fact that the beneficial effects seem to be dependent on the harshness of the animal model used (Beauquis et al., 2013; Herring et al., 2011; Hüttenrauch et al., 2017; Montarolo et al., 2013). Nevertheless, due to its beneficial effects, EE started to be evaluated as a potential therapeutic approach in preclinical models of AD.

1.7. Mouse models of Alzheimer's disease

It is important to notice that currently none of the available mouse models presents all the hallmarks typical of AD. The different existing mouse lines develop Aβ plaques, NFTs, synaptic and neuronal loss and behavioural deficits to a variable degree and in different pathological combinations. For example, brain atrophy and neuronal loss is present only in a few mouse models (Wirths and Zampar, 2020), while NFT pathology is seen only when human tau is genetically expressed.

In the initial AD mouse models, researchers focused their attention on the *APP* and *PSEN* mutations discovered in EO-FAD cases. The first mouse model of AD presenting a human *APP* transgene that carries the Indiana mutation (V717F) was developed in 1995 by Games et al. (Games et al., 1995). This group used the platelet derived growth factor-*β* (PDGF) promoter that is highly expressed in the CNS to drive transgene expression. Using this

approach, an overexpression of human APP protein and a proportionate human Aβ accumulation was obtained. Extracellular Aβ deposition started at 6 months of age and increased noticeable by 12-15 months (Reilly et al., 2003). These mice also developed gliosis and dystrophic neurites, together with age-related behavioural deficits (Chen et al., 2000) and synapse loss (Dodart et al., 2000).

At the same time, Hsiao et al. (Hsiao et al., 1996) generated the Tg2576 mouse model, which overexpressed human *APP* with the Swedish mutation (K670N/M671L). Similar to the PDAPP mice, the Tg2576 presented with age-dependent Aβ accumulation that started at 11-13 months. They further showed deficits in spatial memory at around 9-10 months, detected by the MWM task. Subsequently, many other transgenic mouse lines overexpressing human *APP* containing one or more mutations, such as the J20 (Mucke et al., 2000) or APP23 lines (Lalonde et al., 2002; Snellman et al., 2013; Sturchler-Pierrat et al., 1997) were generated. All of these mice presented the same key pathological characteristics of AD such as a high level of Aβ, extracellular plaques, activated microglia and reactive astrocytes, together with behavioural impairment to a variable degree (Codita et al., 2006; Games et al., 2006; McGowan et al., 2006).

In parallel, transgenic mice having mutations in the presenilin genes were generated (Flood et al., 2002; Guo et al., 1999; Nakano et al., 1999). Interestingly, mice that carry only mutations in *PSEN1/2* genes did not develop amyloid plaques, but when crossed with plaque-developing APP lines, the presence of the *PSEN* mutations caused earlier and increased plaque deposition (Holcomb et al., 1998). Different PS1/APP bigenic mice lines were developed, such as the APP/PS1ΔE9 (Borchelt et al., 1997), APP/PS1KI (Casas et al., 2004) and 5xFAD mice (Oakley et al., 2006).

All the transgenic AD mouse models discussed above are generated by the overexpression of mutant genes that cause AD only in the EO-FAD form of the disease, a small portion of patients. During the last years, also lines that try to mimic the sporadic form of AD, such as

the Tg4-42 (Bouter et al., 2013) or the TBA42 model (Wittnam et al., 2012) have been developed. Details regarding the 5xFAD and Tg4-42 mouse model which are relevant within this thesis are discussed in one of the next sections.

1.7.1. The Tg4-42 mouse model

The Tg4-42 mouse model was engineered to express only the $A\beta_{4-42}$ peptide variant, which is one of the most abundant forms of N-truncated Aβ species found in human AD brain (Lewis et al., 2006; Masters et al., 1985b; Portelius et al., 2010). To ensure the production and the secretion in a neuron-specific manner, the genetic construct consists of the $A\beta_{4-42}$ sequence fused to the murine thyrotropin-releasing hormone (TRH) signal peptide under the control of the murine Thy1 promoter (Bouter et al., 2013) (Figure 6). The transgene is predominantly expressed in the CA1 region of the hippocampus and Aβ peptides can be detected at around 2 months of age. The Tg4-42 mouse model does not present with overt extracellular amyloid plaque deposition; however, the secreted $A\beta_{4-42}$ peptides form soluble and neurotoxic aggregates.

In line with this, Tg4-42 mice displayed elevated neuroinflammation as indicated by gliosis in the hippocampus starting from two months of age (Bouter et al., 2013). Moreover, Tg4-42 mice develop an age- and gene dose-dependent hippocampal neuronal loss in the CA1 region. At six months of age, homozygous Tg4-42 mice present a ~50% neuron loss (Antonios et al., 2015), together with an impaired dentate gyrus neurogenesis when compared to age-matched WT mice (Gerberding et al., 2019). In terms of behaviour, these mice display an age-dependent cognitive decline as assessed by spatial (Morris water Maze) and recognition memory tests (novel object recognition), starting at six months of age in the homozygous and at twelve months in heterozygous mice (Bouter et al., 2013; Stazi and Wirths, 2021a). Recently, motor deficits as assessed by the balance beam and rotarod tasks

were also reported (Wagner et al., 2019). Despite the massive CA1 neuronal loss and the cognitive impairment observed in this line, Tg4-42 mice are still susceptible to pharmacological treatment (Antonios et al., 2015) or cognitive stimulation (Hüttenrauch et al., 2016a). As the Tg4-42 mouse model does not overexpress human *APP* or any EO-FAD mutations, it can be regarded as a suitable mouse model to study novel preventative and/or therapeutic strategies for the sporadic form of AD.

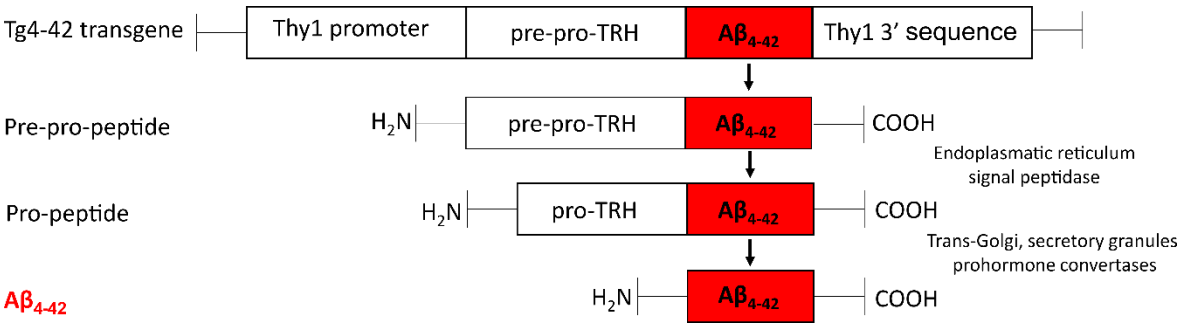

Figure 6: Schematic drawing of the Tg4-42 transgene.
In Tg4-42 mice, the transgenic Aβ4-42 construct is fused to pre-pro-TRH to ensure its secretion within the secretory pathway. The Thy1 promoter ensures expression in a neuron-specific manner. In the Trans-Golgi, the Aβ4-42 construct is cleaved by the prohormone convertase and becomes secreted. Figure generated after (Alexandru et al., 2011; Wittnam et al., 2012).

1.7.2. The 5xFAD mouse model

The double transgenic 5xFAD mouse model was first described in 2006 by Oakley et al. (Oakley et al., 2006) and harbours mutations of both the *APP* and the *PSEN* genes under the control of the Thy1 promoter. In total, 5xFAD mice co-express five EO-FAD mutations; the human APP695 isoforms carrying the Florida (I716V), London (V717I) and Swedish (K670N/M671L) mutations and the PSEN-1 transgene with the M146L and L286V mutations (Oakley et al., 2006) (Figure 7). Whilst the London, Florida and both PSEN mutations lead to an increase in the formation of $A\beta_{x-42}$, the Swedish mutation additionally increases the levels of total Aβ. Different reports point to sex differences in terms of APP and Aβ accumulation (Maarouf et al., 2013; Oakley et al., 2006), with the females having higher APP levels and increased amyloid formation compared to males. This difference might be due to an estrogen response to the Thy1 promoter (Sadleir et al., 2015, 2018). Aβ

pathology appears as early as at six weeks of age, when intracellular Aβ starts to accumulate in the cortical layer V and in the subiculum of the hippocampus (Oakley et al., 2006). In 5xFAD the intraneuronal Aβ deposition correlates well with neuronal loss (Eimer and Vassar, 2013), and astro- and microgliosis (Jawhar et al., 2012; Oakley et al., 2006) in 5xFAD mice. In addition to the typical neuropathological characteristics of AD, 5xFAD mice develop progressive age-dependent behaviour and motor deficits. Jawhar et al. (Jawhar et al., 2012) as well as Devi and Ohno (Devi and Ohno, 2010, 2013) independently described deficits in working, spatial and episodic memory together with decreased anxiety levels and an impairment of motor function. Therefore, this mouse model summarizes the main hallmarks of the AD neurodegeneration progression and represents a useful instrument to study both the molecular mechanisms and possible therapeutic approaches to fight the disease.

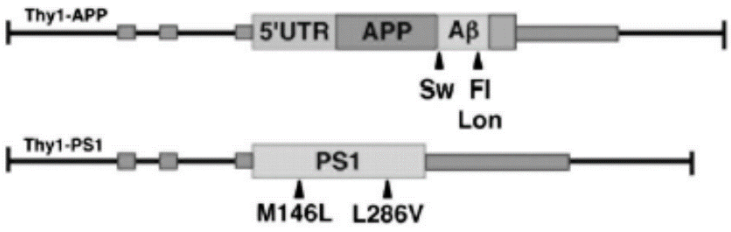

Figure 7: Schematic figure of the 5xFAD transgene.
The 5xFAD mouse model overexpresses both human APP695 and PS1 transgene under the control of the murine Thy1 promoter. The mutations within the two transgenes are indicated by arrow-heads. Sw=Swedish, Lon=London and Fl=Florida. Figure taken with permission from (Oakley et al., 2006) "Copyright 2006 Society for Neuroscience".

1.8. Project objectives

Until today, no effective therapeutic options to treat Alzheimer's disease have been identified. Therefore, the focus of many current research efforts moved to preventative and non-pharmacological strategies to delay or even stop the progression of the cognitive decline observed in AD patients. The aim of the present doctoral thesis is to investigate different kinds of pharmacological and non-pharmacological preventative strategies in two different

mouse models of AD; the widely established 5xFAD and the more recent Tg4-42 model, representing a mouse model for the sporadic form of AD as it overexpresses only an Aβ variant without EO-FAD APP mutations.

To summarize, the objectives of the present work are the following:

- **Project I, project II, and project III: The effect of long-oral memantine, caffeine or riluzole treatment on pathological changes in Tg4-42^{hom} mice**
  - ❖ Does chronic oral memantine, caffeine, or riluzole treatment prevent motor and memory deficits of Tg4-42^{hom} mice?
  - ❖ Can the severe CA1 neuron loss in Tg4-42^{hom} mice be rescued upon memantine, caffeine, or riluzole treatment?
  - ❖ Does memantine, caffeine, or riluzole restore the impaired neurogenesis of Tg4-42^{hom} mice?
- **Project II part II: The effect of long-term oral caffeine treatment on the pathology 5xFAD mice**
  - ❖ Does long-term oral caffeine treatment improve spatial memory impairment of 5xFAD mice?
  - ❖ Does caffeine-treatment have an impact on amyloid deposition, inflammatory status, and APP processing in brains of 5xFAD mice?
- **Project IV: Physical activity and cognitive stimulation ameliorate learning and motor deficits in Tg4-42^{hom} mice**
  - ❖ Does housing in a stimulating environment influence the sensory-motor performance of the Tg4-42^{hom} mouse model?
  - ❖ Does physical exercise and a stimulating environment ameliorate working, recognition and spatial memory of the Tg4-42^{hom} mouse?

2. MATERIAL AND METHODS

2.1. Reagents and Kits

The chemicals, reagents and kits used are listed in Table 1.

Table 1: Reagents and kits

Reagent	Manufacter	Type
Acetic acid	Merck, Darmstadt, Germany	Reagent
Agarose	Lonza, Basel, Switzerland	Reagent
Aqua	Braun, Melsungen, Germany	Reagent
Benzonase	Merck, Darmstadt, Germany	Reagent
Boric acid	Sigma-Aldrich, St. Louis, USA	Reagent
Bovine serum albumin (BSA)	Roth, Karlsruhe, Germany	Reagent
Citric acid	Roth, Karlsruhe, Germany	Reagent
Complete Mini-Phosphatase Inhibitor Tablets	Roche, Basel, Switzerland	Reagent
Complete Mini-Protease Inhibitor Tablets	Roche, Basel, Switzerland	Reagent
DAB Substrate Kit, Peroxidase (HRP), with Nickel, (3,3'-diaminobenzidine)	Vector Laboratories, Burlingame, USA	Kit
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe, Germany	Reagent
DNA ladder 100 bp	Bioron, Ludwigshafen, Germany	Reagent
dNTPs	Invitrogen, Carlsbad, CA, USA	Reagent
Dulbecco's Phosphate Buffered Salt Solution (DPBS)	Bio & SELL GmbH, Nuremberg, Germany	Reagent
Ethanol 99%	Chemsolute,Renningen, Germany	Reagent
Ethylendiaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, USA	Reagent
Fetal Calf Serum (FCS)	Biochrom, Berlin, Germany	Reagent
Formic Acid, 98%	Roth, Karlsruhe, Germany	Reagent
Haematoxylin Solution	Roth, Karlsruhe, Germany	Reagent
HD green plus DNA stain	Intas,Göttingen, Germany	Reagent
Histofix solution containing 4% formalin	Roth, Karlsruhe, Germany	Reagent

Human (6E10) Abeta Peptide Ultra-Sensitive Kit	Meso Scale Discovery, Rockville, USA	Kit
Hydrochloric acid (HCl), 37%	Roth, Karlsruhe, Germany	Reagent
Hydrogen peroxide (H2O2)	Roth, Karlsruhe, Germany	Reagent
Isopropanol	Roth, Karlsruhe, Germany	Reagent
Ketamin 10%	Medistar, Ascheberg, Germany	Reagent
Methanol	AppliChem, Darmstadt, Germany	Reagent
MgCl2 (25 mM)	Axon, Kaiserslautern, Germany	Reagent
Molecular-grade water	Braun, Melsungen, Germany	Reagent
Non-fat Dry Milk Powder	Roth, Karlsruhe, Germany	Reagent
Paraffin	Roth, Karlsruhe, Germany	Reagent
Paraformaldehyde (PFA)	Roth, Karlsruhe, Germany	Reagent
PCR 10X reaction buffer	Axon, Kaiserslautern, Germany	Reagent
Pierce BCA Protein assay Kit	Thermo Fisher Scientific, Waltham, USA	Kit
Proteinase K	Peqlab, Erlangen, Germany	Reagent
Roti-Histokitt	Roth, Karlsruhe, Germany	Reagent
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany	Reagent
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany	Reagent
Sodium hydroxide (NaOH)	AppliChem, Darmstadt, Germany	Reagent
Sucrose	Roth, Karlsruhe, Germany	Reagent
Taq polymerase	Axon, Kaiserslautern, Germany	Reagent
Tris(hydroxymethyl)aminomethane (Tris)	Roth, Karlsruhe, Germany	Reagent
Triton X-100	Roth, Karlsruhe, Germany	Reagent
Tween 20	Roth, Karlsruhe, Germany	Reagent
Vectastain ABC Kit	Vector Laboratories, Burlingame, USA	Kit
Xylazine (Xylariem)	Ecuphar, N.V. Oostkamp, Belgium	Reagent
Xylene	Roth, Karlsruhe, Germany	Reagent

2.2. Laboratory animals

2.2.1. Care and general conditions

All animals were handled according to the German guidelines for animal care and the guidelines of the "Federation of European Laboratory Animal Science Association" (FELASA).and all experiments have been approved by the local animal care and use committee (Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES, Lower Saxony). Animals were kept on a 12 hour/12 hour inverted dark/light cycle (light phase between 8 PM and 8 AM) and mice were sacrificed immediately after the last day of testing. All behaviour experiments were performed during the dark phase. Access to food and water was provided ad libitum. In this study both genders were used. The same control groups (WT and Tg4-42 SH) were used throughout the experiments.

2.2.2. Transgenic mice

2.2.2.1. Tg4-42

Generation of the Tg4-42 mouse model has been published previously (Bouter et al., 2013). In brief, the Tg4-42 mouse model uses the murine Thy1-promotor to overexpress a genetic construct comprising the human $A\beta_{4\text{-}42}$ sequence fused to the murine thyrotropin-releasing hormone (TRH) signal peptide, allowing Aβ secretion. Tg4-42 mice were generated and maintained on a C57Bl/6J (wild-type, WT) genetic background. In this study, only homozygous animals, $Tg4\text{-}42^{hom}$ (Tg4-42 in the figure of the results section 3) were used.

2.2.2.2. 5xFAD

The generation of 5xFAD mice (Tg6799) has been previously described (Oakley et al., 2006). In brief, 5xFAD mice have been backcrossed for more than ten generations to WT

mice from the Jackson Laboratory (Bar Harbor, ME, USA) and were maintained on a C57Bl6 genetic background as heterozygous transgenic line. The 5xFAD mice overexpress both the human APP695 amino acid isoform (carrying the Swedish, Florida and London mutation) and the human presenilin-1 (*PSEN-1*) gene, carrying the M146L and L286V mutation, under the control of the murine Thy1 promoter.

2.2.3. Housing conditions and pharmacological treatments

2.2.3.1. Standard Housing

Mice kept under standard housing (SH) conditions were housed in standard rodents individually ventilated cages (33 cm x 18 cm x 14 cm) in groups of 3-4 animals until the age of 6 months. The cages were equipped with bedding and nesting material and cleaned once per week to ensure animals welfare.

2.2.3.2. Enriched environment housing

Mice that were housed under environmental enrichment (EE) conditions were transferred to larger rat cages (55 cm x 34 cm x 20 cm) at 2 months of age until the age of 6 months. EE cages were equipped with colourful toys, tunnels, houses, nesting material, and sawdust bedding which were cleaned and re-arranged weekly to increase the sense of novelty (Hüttenrauch et al., 2016a). Additionally, the cages were equipped with two different types of running wheels to promote physical activity on a voluntary basis. Mice were kept in groups of 3 - 4 animals to ensure social interaction.

2.2.3.3. Pharmacological treatments

In this study, three different drugs were used: caffeine (Sigma-Aldrich, 300mg/L), memantine hydrochloride (Heumann, 10mg/ml) and riluzole (Santa Cruz Biotechnology, 25mg/L). Chronic pharmacological treatments were initiated at two months of age until six months and all the substances were administered orally via the drinking water.

2.3. Behavioural testing

Behavioural testing timeline is summarized in Figure 8. To diminish odour cues, all apparatus were cleaned after each mouse and trials using 70% ethanol solution.

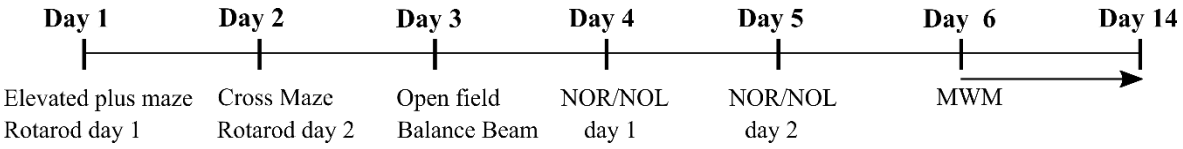

Figure 8: Behavioural testing timeline.
At six months of age, mice were analysed using a battery of motor and learning memory tasks for fourteen days with ongoing treatment or EE housing.

2.3.1. Anxiety phenotype assessment

2.3.1.1. Elevated plus maze

The elevated plus maze (EPM) is a well-established test to assess anxiety levels (Jawhar et al., 2012). The EPM test is based on the aversion of mice to explore open and elevated spaces and the curiosity to explore novel environments (Karl et al., 2003). The EPM apparatus consisted of four arms of 15-cm length and 5-cm width in a “plus” configuration, raised 75 cm above a padded surface. Two oppositely positioned arms contained lateral walls (closed arms), whereas the other pair of arms were opened (open arms). Experiments were carried out under red light conditions, and mice were placed in the central area of the apparatus facing one of the open arms, allowing mice to explore the maze freely for a single 5 minutes trial. Distance travelled, average speed, arm entries, and percentage of time spent in each

arm were recorded and calculated with the ANY-Maze tracking software (Stoelting Europe, Ireland, Dublin). Anxiety-like behaviour was calculated by measuring the time spent in the open arms, with longer times spent in the open arms corresponding to reduced anxiety levels (Karl et al., 2003).

2.3.1.2. Open field

The open field (OF) test was used to assess locomotors activity, exploratory behaviour, and anxiety levels as described previously (Jawhar et al., 2012). Moreover, the OF test was used as the habituation phase for the following novel object recognition and novel object localization tests. During the OF test, mice were placed into a square grey plastic box (50 cm x 50 cm), which they were allowed to explore freely for 5 min. ANY-Maze video tracking software was used to record the percentage of time spent in the central part of the arena and total distance travelled during the single 5-min trial.

2.3.2. Motor phenotype assessment

2.3.2.1. Accelerating rotarod

Motor performance and motor learning skills were examined by the rotarod test (Shiotsuki et al., 2010). The rotarod consists of an apparatus linked to a corresponding computer software (TSE, Technical and Scientific Equipment) that records the time point at which the mouse crosses a photo sensor underneath the rotator. The test was performed under red light conditions on two consecutive days with four trial sessions each day. Mice were allowed to rest for at least 15 minutes between the trials. Each mouse was placed onto the rotarod facing the back of the apparatus and allowed to remain on the rotator for a maximum time of 300 seconds. During that time, the speed continuously increased from 4 revolutions per minute

(r.p.m.) to 40 r.p.m. Trials were completed when either animals fell off or the maximal time was reached, and the time spent on the rod was recorded as an indicator of motor performance (latency to fall [s]).

2.3.2.2. Balance beam

The balance beam task was used to assess balance and motor coordination as described previously (Wirths et al., 2008). A 50 cm long wooden beam (1 cm Ø) was clamped to two plastic escape platforms (9 cm × 15 cm), 44 cm above a padded surface. The mouse was placed on the centre of the beam facing one of the two platforms, and each animal was given three trials during a single day of testing. The time the animal stayed on the beam was recorded and the resulting time spent on the beam of all three trials was averaged. If an animal remains on the beam for the entire 60 s trial period or escaped to one of the two platforms, the maximum time of 60 s was recorded (latency to fall [s]). The beam was cleaned with 70% ethanol between each mouse as well as between trials to avoid odour cues.

2.3.3. Memory and cognition phenotype assessment

2.3.3.1. Cross Maze

Working memory and spontaneous alternation rates were assessed using the cross-maze test (Jawhar et al., 2012). The apparatus consists of four arms arranged in a "+" configuration extending from a central space measuring 8 cm x 8 cm (arm sizes: 30 cm length, 8 cm width, and 15 cm height). During the 10 minutes test trial, each mouse was placed at the end of one arm facing the wall and allowed to move freely through the maze. A correct alternation was defined as consecutive entries into the four arms in overlapping quadruple sets without re-visiting a previous arm (e.g., 1, 3, 2, 4, or 1, 2, 3, 4, but not 4, 1, 3, 1). Alternation frequency

was calculated as the ratio of correct to total alternations (total number of arm entries -3) according to the formula: $\frac{(correct\ alternations\ x\ 100)}{total\ alternations}$.

The ANY-Maze software package (Stoelting Europe) was used to record and automatically evaluate the number and order of arm entries as well as the total distance travelled.

2.3.3.2. Novel Object Recognition

The Novel Object Recognition (NOR) is a widely used test to assess recognition memory and novelty preference in rodents (Antunes and Biala, 2012). The NOR test was performed on three consecutive days in an open field box (grey plastic, non-translucent, 50 cm x 50 cm). On the first day (habituation phase), each animal performed the OF test as described in section 2.3.1.2. Twenty-four hours after the OF, two identical objects were placed in the same box. Mice were allowed to freely explore the objects for 5 min (exploration phase) and were returned to their home cage. Another 24 hours later, one of the objects was exchanged with a novel object consistent in height and volume but different in shape and appearance, and once again, animals were allowed to freely explore the items for 5 min (testing phase).

2.3.3.3. Novel Object Localization

The novel object location (NOL) memory task evaluates spatial memory and is based on the ability of mice to recognize when a familiar object has been relocated (Denninger et al., 2018). Twenty-four hours after the OF (for details see section 2.3.1.2) during the training phase, mice freely explore two duplicate objects (O_1 and O_2) for a period of 5 min, which were placed close to the far corners of the square arena. After a delay of 24 h, one object was relocated in the diagonally opposite corner. Thus, both objects in the testing phase were equally familiar, but now one was presented in a new location (De Pins et al., 2019).

For both memory tests (NOR and NOL), object exploration was scored whenever the mouse sniffed the objects when looking at them. In contrast, climbing onto the object was not considered as exploration as opposed to playing with them (Leger et al., 2013). Data collection and video analysis were performed blind to the experimental condition and genotype. The percentage of exploration time for the novel object/novel location and discrimination indices (DI) were calculated as follows:

$$\%\ exploration\ time = \frac{time\ at\ novel\ x\ 100}{total\ exploration\ time} \quad \text{and} \quad DI = \frac{time\ at\ novel - time\ at\ familiar}{total\ exploration\ time}.$$

2.3.3.4. Morris Water Maze

The Morris Water Maze test (MWM) (Morris, 1984) was used to assess spatial reference memory as previously described (Bouter et al., 2013). In brief, mice are trained to learn to find a submerged platform (ø 10 cm) in a circular pool (ø 110 cm) filled with water and made opaque with non-toxic white paint. Initially, a "cued training", was carried out on three consecutive days (four trials per day), in which the submerged platform was marked with a triangular flag. Twenty-four hours after the last trial of the cued training, mice performed five days of "acquisition training" (four trials per day), in which proximal cues were added around the pool and the triangular flag was removed from the platform. During the acquisition-training phase, the platform remained in the same position for each mouse. A "probe trial" was conducted to assess spatial reference memory 24 hours after the last trial of the acquisition training. During this 1 min trial, the platform was removed, however, proximal and distal cues remained in the same position as in the acquisition phase. Since the platform location was kept constant during the acquisition training, mice with intact spatial reference memory should exhibit a target quadrant preference. Between the trials, mice were dried and kept under infrared light to prevent hypothermia. All trials were recorded using a video tracking software (ANY-maze, Stoelting Europe) and parameters such as escape

latency, swimming speed, swimming path quadrant preference, latency to first entry into the platform/target quadrant, time in the platform/target quadrant and entries into the platform/target quadrant were evaluated.

2.4. Tissue collection and preservation

Brain tissues were collected and preserved in different ways, depending on the subsequent analysis. Mice were deeply anesthetized, using a solution of ketamine (10% stock solution) and xylazine (23.3 mg/ml) diluted in molecular-grade water, and transcardially perfused using ice-cold 0.01 M phosphate-buffered saline (PBS, 10X PBS contains 1,37 M NaCl, 27 mM KCL, 100 mM Na_2HPO_4 and 18 mM KH_2PO_4, Bio & SELL GmbH). After that, brains were carefully dissected, the fur and skin surrounding the skull were removed and a series of incisions were made to remove the brain. First, an incision was made from the occipital hole towards the anteriorly positioned olfactory bulbs, followed by another on the level of the olfactory bulbs. Next, the skull was removed, and the brain was extracted and transferred to a glass dish on ice. The brain was separated at the sagittal midline and the right hemisphere (RH) was placed in embedding cassettes (Simport) and post-fixed in 4% formalin solution at 4 °C for at least 72 h protected from light before embedding in paraffin for immunohistochemistry (see section 2.5.2). The left hemisphere (LH) was post-fixed in 4% paraformaldehyde (PFA) in 0.01 M PBS for at least 24 h before being transferred to a 30% sucrose solution (in 0.01 PBS) for cryo-protection. For RNA and protein isolation, further dissections were made, following removal of the cerebellum and olfactory bulb, the hippocampus and the cortex of each hemispheres were isolated, kept on dry ice and stored at -80°C until further use.

2.5. Biochemistry and molecular biology

2.5.1. Genotyping of transgenic mice

2.5.1.1. Isolation of genomic DNA

Genomic DNA was isolated from ear puncture biopsies to genotype transgenic mice before any further use. Ear punctures of the mice were collected after the weaning at the animal facility of the University Medical Centre, Göttingen. Each biopsy sample was kept in a 1.5 mL Eppendorf tube at -20° until DNA isolation. For this purpose, 200µL of 1x lysis buffer (200 mM Tris/HCl pH 8.5, 10 mM EDTA, 0.4% dodecyl sulphate (SDS), 400 nM NaCl) supplemented with 2.5-µl proteinase K (20 mg/ml stock, Peqlab, Erlangen, Germany) was added to each ear biopsy. The samples were lysed at 56°C over-night (o/n) under shaking conditions, with 450 r.p.m in a Thermomixer Compact (Eppendorf, Hamburg, Germany). Next, samples were centrifuged at 17,000 r.p.m for 20 min at 4°C (Heraeus Biofuge Stratos) and the resulting supernatant was transferred to a fresh 1.5 mL Eppendorf tube containing 200 µL cold isopropanol. The tubes were inverted gently to allow DNA precipitation and centrifuged at 13,000 r.p.m for 10 min at room temperature (RT). The supernatant was carefully discarded, and the pellet washed three time with ice-cold 70% ethanol followed by another centrifugation step (13,000 r.p.m, 10 minutes, RT). After the last ethanol-washing step, the supernatant was carefully discarded, and the pellet was dried in the Thermomixer at 56°C for 40 min. Finally, the genomic DNA pellet was dissolved in 30 µL molecular-grade water (Aqua, Braun, Germany) at 55°C overnight in a Thermomixer Compact before being stored at 4°C until further usage.

2.5.1.2. Polymerase chain reaction (PCR)

Mice carrying the desired transgene were identified via conventional polymerase chain reaction (PCR). Genomic DNA samples, isolated as described in the previous section, were

used for genotyping. The different reactions mix were prepared according to Table 2 and 3. The PCR was run in a Lab Cycler (SensoQuest, Göttingen) thermocycler. The PCR cycling program is outlined in Table 4.

Table 2: Reaction mix for Tg4-42 genotyping

Reagent	Volume (µL)
ddH20	11.2
10X Reaction buffer	2.0
MgCl2 (25 mM)	1.6
dNTPs (2 mM)	2
Aβ3-42 for primer	1.0
Aβ3-42 rev primer	1.0
Taq polymerase (5 U/µl)	0.2
DNA	1.0
Total volume per sample	20

Table 3: Reaction mix for 5xFAD genotyping

Reagent	Volume (µL)
ddH20	10.6
10X Reaction buffer	2.0
MgCl2 (25 mM)	3.2
dNTPs (2 mM)	2.0
hAPP for primer	0.5
hAPP rev primer	0.5
Taq polymerase (5 U/µl)	0.2
DNA	1.0
Total volume per sample	20

Table 4: PCR genotyping program for Tg4-42 and 5xFAD

Step	Temperature	Duration
1	94°	2 min
2	94°	45 seconds
3	58°	1 min
4	72°	1 min
5	Repetition of steps 2-4	40 cycles
6	72°	5 min
7	4°	∞

PCR products were analysed using agarose gel electrophoresis. To prepare a 2 % agarose gel, 35-ml of 1xTBE buffer (1L 10x TBE solution: 108 g Tris, 55 g boric acid, 40 ml 0.5 M Na_2EDTA (pH 8.0) was mixed with 0.7 g agarose (Lonza, Basel, Switzerland) and boiled in a microwave until complete dissolution of the agarose. Next, 2 µl HDGreen® Plus Safe DNA Dye (Intas Science Imaging Instruments GmbH, Göttingen, Geramany) were added to the agarose solution and the mixture was transferred into a casting tray with a comb to form the wells. Once the gel became solid, the comb was removed and the gel was placed in an electrophoresis chamber (Serva) containing 150 ml of 1xTBE buffer. Ten µl of the PCR product were mixed with 2 µl of 10X loading buffer and added into the wells. For size estimation, a volume of 5 µL 100 bp DNA ladder (Bioron, Ludwigshafen, Germany) was loaded in the first line. The gel was run for 30 min at 135 V and visualized under UV light using the SERVA BlueCube 300 (Serva).

2.5.1.3. Primers

All primers were acquired from Eurofins (Ebersberg) and used at a 1:10 dilution from a stock with a 10 pmol/µl concentration (100 pmol/µl primer stock prepared in molecular-grade water). Sequences of the primers used are listed in Table 5.

Table 5: Primer used for PCR

Name	Sequence 5'→3'
Aβ3-42 for	TCCGGCCAGAACGTCGATTC
Aβ3-42 rev	GGAGAAGCAAGACCTCTGC
hAPP for	GTAGCAGAGGAAGAAGTG
hAPP rev	CATGACCTGGGACATTCTC

2.5.2. Protein isolation from mouse hippocampi and cortices

Frozen hippocampi and cortices were weighed and homogenized in 0.7 ml TBS buffer (120 mM NaCl, 50 mM Tris, pH 8.0 including complete mini-Protease Inhibitor tablets and complete mini-Phosphatase Inhibitor Tablets, Roche) per 100 mg tissue using a sonicator (Sonitrome, tip 1.5 MS, amplitude 80%, time 30 sec, and pulse 0,05 sec - 1 sec). The resulting homogenate was centrifuged at 17,000 x g for 20 min at 4°C (Heraeus Biofuge Stratos). The supernatant (TBS-soluble proteins fraction) was stored at -80°C. The remaining pellet was dissolved in 0.8 ml (cortices) and 0.2 ml (hippocampi) of 2% SDS with complete protease inhibitors and sonicated again, followed by a centrifugation step of 17,000 x g for 20 min at 4°C. To remove residual DNA, the supernatant (SDS-soluble proteins fraction), was transferred to a new Eppendorf tube containing 1 µl of benzonase and was gently mixed and incubated for 5 min at RT followed by a final centrifugation step (17,000 x g for 20 min at 4° C). The SDS-soluble protein fraction was aliquoted and stored at -80°C until further use.

2.5.2.1. Determination of protein concentration

Protein concentrations were determined using the Pierce BCA Protein assay Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. Briefly, a stock solution of 2 mg/ml albumin standard (BSA) was diluted for the generation of a standard curve. Twenty-five µl of protein samples as well as BSA standard dilutions were loaded in triplicates into a 96-well plate. Reagent A and Reagent B were mixed in a ratio of 50:1 and added to each well in a total volume of 200 µl. The samples were gently mixed on a 96-well plate mixer for 30 sec and incubated at 37°C for 30 min in the dark. Protein concentrations were measured at 592 nm using a µQuant plate reader (BioTek Instruments, Inc.) and analysed with the MikroWin 2000 software (v4.04, Mikrotek).

2.5.2.2. Enzyme-linked immunosorbent assay (ELISA) analysis

$A\beta_{1\text{-}42}$ peptide levels were determined in the SDS-fractions of cortex and hippocampal homogenates using a sandwich enzyme linked immunosorbent assay (ELISA) as described in (Walter et al., 2019). Briefly, to detect full-length $A\beta_{1\text{–}42}$ peptides, monoclonal antibody IC16 (Antonios et al., 2014) was used as a capture antibody and combined with $A\beta_{42}$ C-terminus-specific detection antibody BAP-15 (Brockhaus et al., 1998; Hahn et al., 2011). A standard curve was generated with synthetic $A\beta_{1\text{-}42}$ peptides (JPT). 96-well high-binding microtiter plates (Greiner Bio-One) were incubated overnight at 4 °C with the capture antibody in PBS (pH 7.2). After excess capture antibody was removed, freshly diluted brain samples or $A\beta_{1\text{-}42}$ peptide standards (in PBS, 0.05% Tween-20, 0.5% BSA) were added. Then, the detection antibody labelled with horseradish peroxidase using the Pierce EZ-Link Plus Activated Peroxidase kit (ThermoFisher Scientific) and diluted in PBS, 0.05% Tween-20, 0.5% BSA was added to each well and incubated overnight at 4 °C. Plates were washed three times with PBS containing 0.05% Tween-20 and once with PBS. Subsequently, 50 µl

of trimethylbenzidine ELISA peroxidase substrate (ThermoFisher Scientific) was added and incubated for 1–5 min at RT in the dark. The reaction was stopped by adding 50 µl of 2 M H_2SO_4, and the absorbance was recorded using a Paradigm microplate reader (Beckman Coulter) at 450 nm.

2.5.2.3. Western blot- SDS-PAGE

The APP processing was analysed by Western blot assay in TBS- and SDS-soluble hippocampal brain fractions of untreated and caffeine-treated 5xFAD mice. Total protein concentrations were determined as described in section 2.5.15, and equal amounts of protein were separated on 12% Bis–Tris SDS-PAGE gels and transferred onto PVDF membranes (Merck) by electroblotting. The membranes were blocked with 5% milk powder in TBST (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20, pH 7.4) for 1h at RT, and then incubated overnight at 4 °C with the primary antibody diluted in TBST. The following primary antibodies against APP were used: CT-15 (rabbit polyclonal raised against the C-terminal 15 amino acids of human APP, 1:3500) (Walter et al., 2019); 22C11 (mouse monoclonal raised against residues 66–81 of human APP, 1:1000, kindly provided by Dr. Stefan Kins, University of Kaiserslautern, Germany) (Walter et al., 2019); anti-APPs-α (mouse monoclonal, clone 2B3, recognizing the C-terminal neoepitope generated by α-secretase cleavage of APP, 1:50, IBL Cat. No. 11088); anti-Actin (rabbit polyclonal, 1:2000, Sigma-Aldrich Cat. No. A2066); anti-Tubulin (mouse monoclonal, clone DM1A, 1:5000, Sigma-Aldrich Cat. No. T6199). Subsequently, a secondary antibody labelled with a near-infrared fluorescent dye (IRDye 800CW goat anti-mouse IgG or goat anti-rabbit IgG, 1:10000, LI-COR Biosciences) diluted in TBST was added and incubated for 1 h at RT. Fluorescence signals were detected with the Odyssey CLx Imaging System and quantified using the Image Studio Software 2.1 (LI-COR Biosciences).

2.5.3. Immunohistochemistry

2.5.3.1. Paraffin embedding of mouse brain

Samples form right brain hemispheres were collected as described in the previous section (see 2.4). Next, the samples were processed with a TP1020 automatic tissue processor (Leica Biosystems, Nussloch, Germany). First, the tissues were incubated in 4% formalin solution (Roti-Histofix 4%, Roth, Karlsruhe, Germany) until the automatic processing starts. The dehydration phase was carried by transferring the tissue samples into double-distilled water (ddH_20) for 30 min and later into a series of ascending ethanol concentrations (50%, 60%, 70%, 80%, and 90%) for one hour each. Then, the samples were incubated twice with 100% ethanol for 60 min each followed by an incubation in xylol for one hour. Finally, tissue samples were immersed twice in melted paraffin for 60 min each before being embedded in paraffin blocks using an EG1140 H Embedding Station (Leica). Immunohistochemistry was performed on 4 μm thin sagittal paraffin brain samples produced by manual with a sliding microtome (HM335E, Microme). Brain samples were collected on SuperfrostPlus microscopic slides in a 55°C dH_2O bath, dried on a 55°C hot plate for approx. 30 minutes and incubated at 37°C o/n for further drying.

2.5.3.2. 3,3'-Diaminobenzidine (DAB) immunohistochemistry

For DAB immunohistochemistry (IHC) staining 4 μm paraffin sections were used. The slides were deparaffinised and rehydrated using the following protocol: 2 x 5 min 100% xylol treatment followed by a descending alcohol series (100% ethanol (EtOH) for 10 minutes, 95% EtOH for 5 minutes, 70% EtOH for 1 min and a washing step in ddH2O for 1 min). Endogenous peroxidase blocking was done by immerging the slides in a solution of 0.3% H_2O_2 in 0.01 M PBS for 30 minutes. For antigen retrieval, the samples were boiled in 0.01M citrate buffer (pH 6.0) for 10 minutes in a microwave (approximately 2 min at 800 W, then

approximately 8 min at 200 W), and were allowed to cool down for 15 minutes at RT. Then, sections were washed for 1 min in ddH2O, permeabilized for 15 min in 0.01 M PBS +0.1% Triton X-100 (Roth, Karlsruhe, Germany) and washed for 1 min in 0.01 M PBS. To reveal aggregated Aβ peptides, sections were incubated for 3 min in 88% formic acid (Roth, Karlsruhe, Germany), followed by two consecutive washing steps of 5 min in 0.01 M PBS to remove any residual formic acid. Subsequently, the samples were first blocked non-specifically with 0.01 M PBS including 10% fetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA) and 4% milk powder (Roth, Karlsruhe, Germany) for one hour before they were incubated o/n with the respective primary antibody (see Table 7) at RT. On the second day, slides were first washed in 0.1 M PBS with 0.1% Triton X-100 for 15 min, followed by 0.01 M PBS for 1 min. Afterwards, the samples were incubated with the corresponding biotinylated secondary antibody (see Table 8) for 60 min at 37°C. The slides were then washed in 0.01 M PBS for 10 minutes. Next, the sections were incubated in avidin-biotin complex (ABC) solution (VECTASTAIN Elite® ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 90 min at 37°C. The ABC solution was prepared using 0.01 M PBS including 10% FCS; reagents A and reagents B were both diluted 1:100. Prior to the 3,3'-diaminobenzidine (DAB) stain, the slides were once more washed in 0.01 M PBS for 15 min. Using DAB as a chromogen, the staining was visualized. The DAB staining was carried out using the Vector Laboratories DAB Peroxidase (HRP) substrate kit. The developing solution was prepared according to the manufacturer guidelines. Incubation time with DAB was kept constant between each slide. For counterstaining, filtered haematoxylin (Roth, Karlsruhe, Germany) was used and slides were immersed in the solution for 40 sec; remaining haematoxylin was washed away in a one-minute washing step in ddH_20, followed by running tap water for two min. Next, slides were dehydrated in an ascending alcohol series: 1 min in 70% ethanol, 5 min in 95% ethanol, 10 min in 100% ethanol, and 2 x 5 min in 100% xylene. Finally, the slides were embedded using the Roti®-

Histokitt (Roth, Karlsruhe, Germany) as mounting medium and microscope cover slips (Menzel-Glasses, Braunschweig, Germany). The slides were allowed to dry o/n at RT prior to analysis.

2.5.3.3. Free-floating immunohistochemistry

For unbiased stereological estimation of neuron numbers, frozen brain hemispheres (see section 2.4) were first embedded in cutting medium (OCT, optimal cutting temperature embedding medium) for frozen specimen and afterwards coronally cut into 10 series of 30 µm thick sections using a Leica CM1850 UV cryostat. The samples were stored at -80°C until further use. Initially, one brain series was stained in a 12-well net system (Netwell, Costar) using ice-cold 0.01 M PBS for hydration. To ensure a constant floating of the sections, all subsequent steps were carried out on a rotating plate. Next, sections were transferred to 0.3% H_2O_2 in 0.01 M PBS for 30 min to block peroxidase activity. For membrane permeabilization, sections were washed 3 x 10 min in 0.01 M PBS containing 0.1% Triton X-100. Unspecific blocking was done by treatment with 0.01 M PBS containing 10% FCS and 4% milk powder for 1 hour at RT. Primary antibody (see Table 7) was diluted in 0.01 M PBS with 10% FCS and was incubated overnight at RT. On the second day, sections were washed 3 x 10 min in 0.01 M PBS containing 0.1% Triton X-100 followed by one washing step in 0.01 M PBS for 1 min. Then, sections were incubated with the secondary antibody (see Table 8) for 2 hours at RT followed by washing with 0.01 M PBS for 3 x 10 min. Afterwards, sections were incubated in Avidin-Biotin complex (ABC) solution for 90 minutes at RT. After washing 3 x 10 min in 0.01 M PBS, staining was visualized using DAB solution as described in 2.5.2.2. Sections were washed 3 x 10 min in 0.01 M PBS. After washing, sections were mounted in PBS onto Superfrost slides and left to dry o/n. The next day, sections were incubated in 0.01 M PBS for hydration, followed by counterstaining with

filtered haematoxylin for 40 sec. After a short immersion step in ddH_2O, sections were differentiated under running tap water for 5 min. Finally, sections were dehydrated in baths of the following EtOH concentrations: 1 min 70% EtOH; 5 min 95% EtOH; 10 min 100% EtOH followed by 2 x 5 min in xylol and were embedded using Roti® Histokitt mounting medium.

2.5.3.4. Quantification of Aβ plaque load and GFAP immunoreactivity

Extracellular Aβ load was evaluated in the cortex (Co), dentate gyrus (DG), subiculum (Subi) and thalamus (Thal) (Figure 9) in 5xFAD mice using an Olympus BX-51 microscope equipped with a Moticam Pro 282A camera (Motic) and the ImageJ software package (V1.41, NIH, USA). Moreover, intraneuronal Aβ accumulation was quantified in CA1 region of the hippocampus of the Tg4-42^{hom} mice. Serial images of ×100 magnification were captured on three sections per mouse which were at least 30 µm afar from each other. Using ImageJ, pictures were binarized to 8-bit black and white images and a fixed intensity threshold was applied defining the DAB signal. Measurements were performed for a percentage area covered by DAB (Breyhan et al., 2009). Accordingly, for GFAP staining quantification, images of ×200 magnification were captured and the astrocyte-covered areas were analysed as described previously (Saul et al., 2013). The relative Aβ plaque load or GFAP immunoreactivity is expressed with 5xFAD untreated mice or Tg4-42^{hom} SH as the reference parameter.

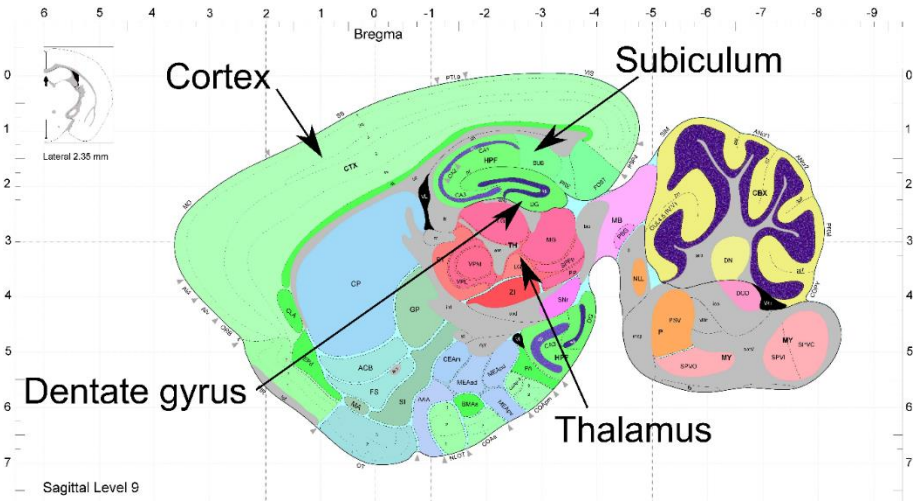

Figure 9: Brain regions, in which the extracellular Aβ plaques load was analysed.
Adapted from Allen Brain Atlas.

2.5.3.5. Primary & secondary antibodies

Primary and secondary antibodies were diluted in 0.01 M PBS containing 10% FCS and are listed in Table 6 and 7, respectively.

Table 6: Primary antibodies

Antibody	Host	Epitope	Isotype	Dilution	Usage	Manufacturer
24311	rabbit	Pan-Aβ	polyclonal	1:500	DAB IHC	Dept. of Psychiatry, UMG (Saul et al., 2013)
D3E10	rabbit	$A\beta_{1-42}$	monoclonal	1:1000	DAB IHC	Cell Signalling
82E1	mouse	$A\beta_{1-16}$	monoclonal	1:1000	DAB IHC	Immuno-Biological Laboratories
GFAP	rabbit	Glial fibrillary acidic protein	polyclonal	1:1000	DAB IHC	Synaptic Systems
DCX	goat	Doublecortin	polyclonal	1:500	free-floating IHC	Santa Cruz Biotechnology
IC16	mouse	$A\beta_{1-15}$	monoclonal	1:250	ELISA	Gift of Dr. S. Weggen

						(Hieke et al., 2010)
BAP-15	mouse	$A\beta_{42}$	monoclonal	1:2000	ELISA	Gift of Dr. K.H Baumann (Brockhaus et al., 1998)
CT-15	rabbit	APP-full length	polyclonal	1:3500	WB	Gift of Dr. Edward Koo (Walter et al., 2019; Weggen et al., 2001)
22C11	mouse	APPs	monoclonal	1:1000	WB	Gift of Dr. Stefan Kins
anti-APPs-α	mouse	s-APP-α	monoclonal	1:50	WB	IBL
anti-Actin	rabbit	Actin	polyclonal	1:2000	WB	Sigma-Aldrich
anti-Tubulin	mouse	Tubulin	monoclonal	1:5000	WB	Sigma-Aldrich

Table 7: Secondary antibodies

Antibody	Host	Conjugated	Dilution	Usage	Manufacture
Anti-mouse	goat	Biotinylated	1:200	DAB IHC	Dianova
Anti-rabbit	goat	Biotinylated	1:200	DAB IHC	Dianova
Anti-goat	rabbit	Biotinylated	1:200	free-floating IHC	Dako
anti-mouse	goat	IRDye 800CW	1:10000	WB	LI-COR Biosciences
anti-rabbit	goat	IRDye 800CW	1:10000	WB	LI-COR Biosciences

2.5.4. Quantification of neuron numbers

2.5.4.1. Haematoxylin staining

Assessment of neuronal loss in the CA1 region of the hippocampus of 6-month-old Tg4-42^{hom} and WT treated or untreated mice (n = 3 for each treatment and genotype) was carried out on sagittal brain sections (Bregma 1.08-1.32) cut as described in 2.5.2.1. Sections were stained with haematoxylin as follows: First, the slides were deparaffinized in xylol (2 x 5 min) and rehydrated in a series of ethanol (10 min 100% EtOH; 5 min 95% EtOH; 3 min

70% EtOH) followed by a 1 min incubation in ddH_2O. Next, sections were counterstained with haematoxylin for 10 min, dipped in tap water, and washed under running tap water for 3 min. Finally, sections were dehydrated using the following incubations steps: 1 min 70% EtOH, 5 min 95% EtOH, 10 min 100% EtOH, 2 x 5 min xylol.

2.5.4.2. Quantification of total neuron numbers in the CA1 area of the hippocampus

Images of the CA1 layer stained with haematoxylin (see 2.5.3.1) were captured at 400x magnifications. The CA1 layer was separated into proximal (extending to CA2) and distal (towards subiculum) parts (Figure 10), and relative neuron numbers setting the WT group as a reference were calculated. Neuronal nuclei were identified according to their size and characteristic appearance clearly differing from glial cells. The number of CA1 neurons in a defined area per section (n = 3 per animal) was counted with the cell counting tool implemented in the ImageJ software package.

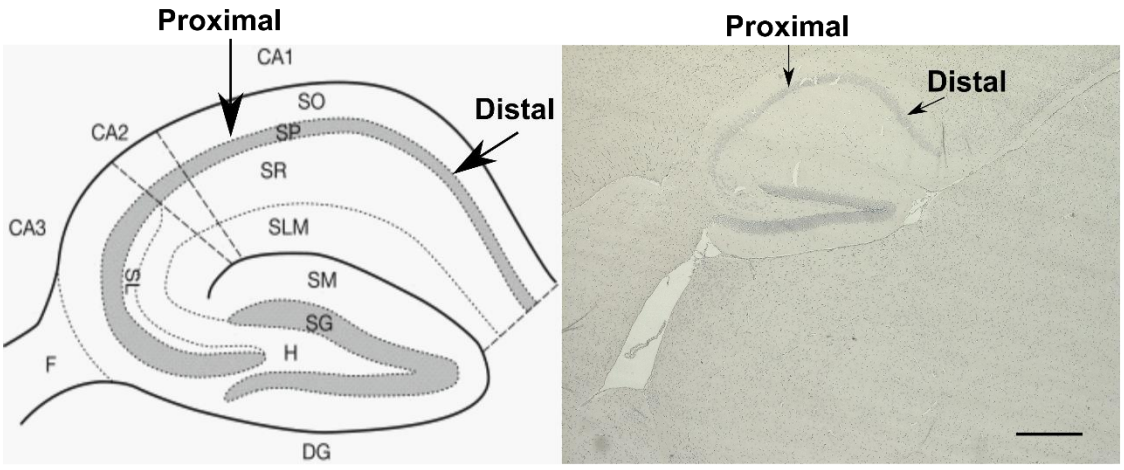

Figure 10: Schematic drawing of hippocampal structure and haematoxylin staining illustrating the CA1 counting area.
CA, cornu ammonis; DG, dentate gyrus; F, fimbria; H, hilus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare; SL, stratum lucidum; SM, stratum moleculare; SG, stratum granulosum. Scale bar: 50µm.

2.5.4.3. Quantification of DCX- positive neuron in the dentate gyrus of the hippocampus

Frozen cryo-protected brain hemispheres were cut into series of 30-µm-thick coronal sections as described in 2.5.2.3. A series of every 10th coronal frozen section was processed in a free-floating staining protocol to quantify the number of new-born neurons (see 2.5.2.3). The total number of new-born neurons was counted in the DG using a stereology workstation and the meander scan option of Stereo Investigator 7 (MicroBrightField, Williston, USA) to quantify all DCX-positive cells in a given section. The resulting neuron number was multiplied by 10 to obtain the total number of new-born neurons per hemisphere.

2.6. Statistical analysis

Details of statistical analysis are given in the respective results section as well as in the figure legends. Differences between groups were analysed with unpaired t-test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test. The number of animals used for behavioural experiments as well as sample sizes used for biochemical analyses or quantification of neuron numbers are given in the figure legends (n). Due to the observation of a floating phenotype in the Tg4-42^{hom} SH mice, six mice from this group (one male, five females) have been excluded from all analyses, except for the floating behaviour (for details see results section 3). All data were given as mean ± standard deviation of the mean (SD). Significance levels were given as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All statistics were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. PROJECT I: The effect of long-term oral memantine treatment on the pathology of the Tg4-42^{hom} mice[1]

Two-month-old WT and Tg4-42^{hom} mice were treated for four months with memantine (20 mg/kg/day) in drinking water (Figure11A). At six months of age, mice were subjected to a battery of behavioural tests for a duration of fourteen days with ongoing oral memantine treatment. At the end of the behavioural testing phase, animals were sacrificed for further biochemical and/or stereological analysis. No differences in water consumption (Figure 11B) or body weight (Figure 11C) among all groups, irrespective of the treatment, were detected.

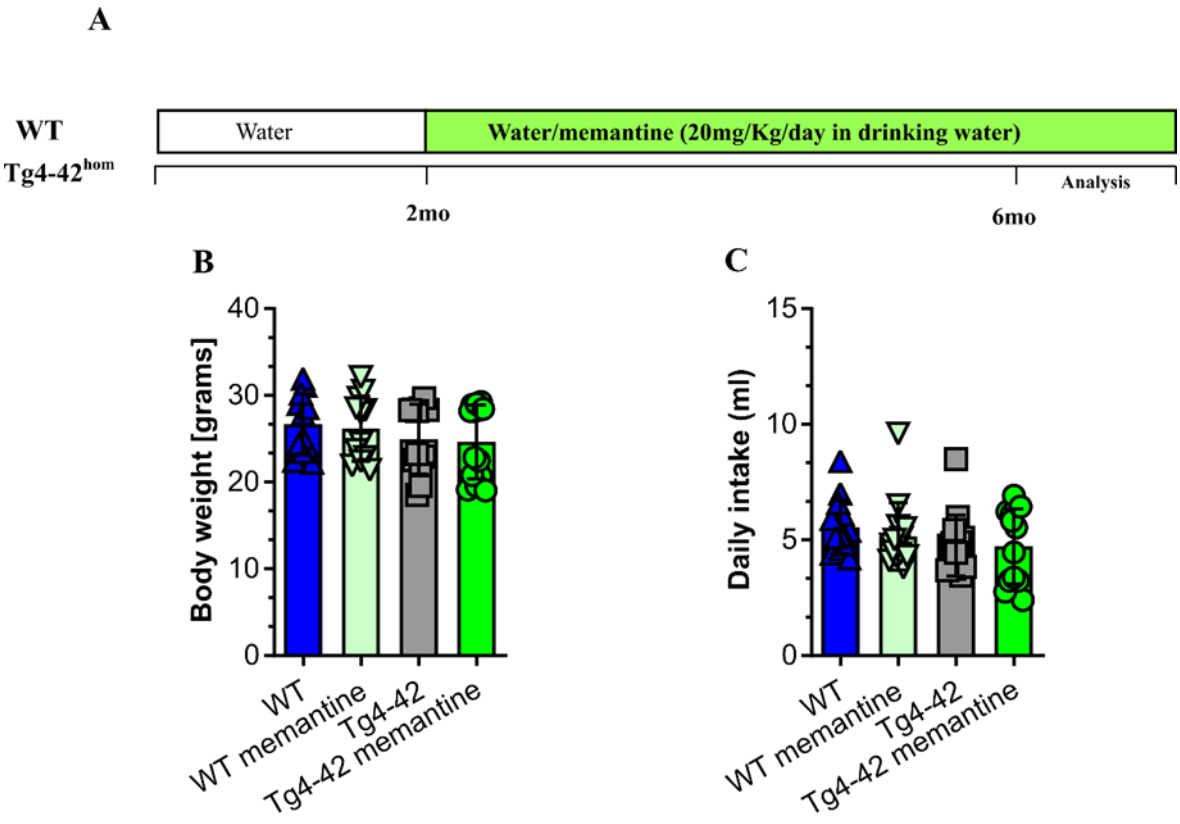

Figure 11: Experimental design and physiological status of animals after memantine treatment.
(A) Experimental design. **(**B) Daily water consumption and body weight assessment during the behavioural test analysis. The daily water intake was similar between the groups, and (C) memantine treatment does not have an influence on the body weight of mice. WT n=12, WT memantine n=14, Tg4-42^{hom} n=12, and Tg4-42^{hom} memantine n=14. Figure adapted from (Stazi and Wirths, 2021a).

[1] The results presented in this chapter are published in **Stazi M**, Wirths O. Chronic Memantine Treatment Ameliorates Behavioral Deficits, Neuron Loss, and Impaired Neurogenesis in a Model of Alzheimer's Disease. Mol Neurobiol. 2021, 58(1): 204-216.

3.1.1. Chronic memantine treatment did not alter anxiety levels in Tg4-42^{hom} mice

To assess if memantine influences the anxiety status of the mice, the EPM test was used. At 6 months of age, Tg4-42^{hom} showed significantly reduced anxiety levels compared with age-matched WT animals (Figure 12A, $p < 0.01$). Chronic memantine treatment could not revert this altered anxiety phenotype, as shown by the percentage of time spent in the open arms (Figure 12A). Calculating the ratio of open arm entries to total arm entries confirmed this result with a significantly higher ratio in Tg4-42^{hom} compared with WT control animals (Figure 12B, $p < 0.05$). No significant difference could be detected in both analyses in the memantine-treated Tg4-42^{hom} compared to untreated mice (Figure 12A and B).

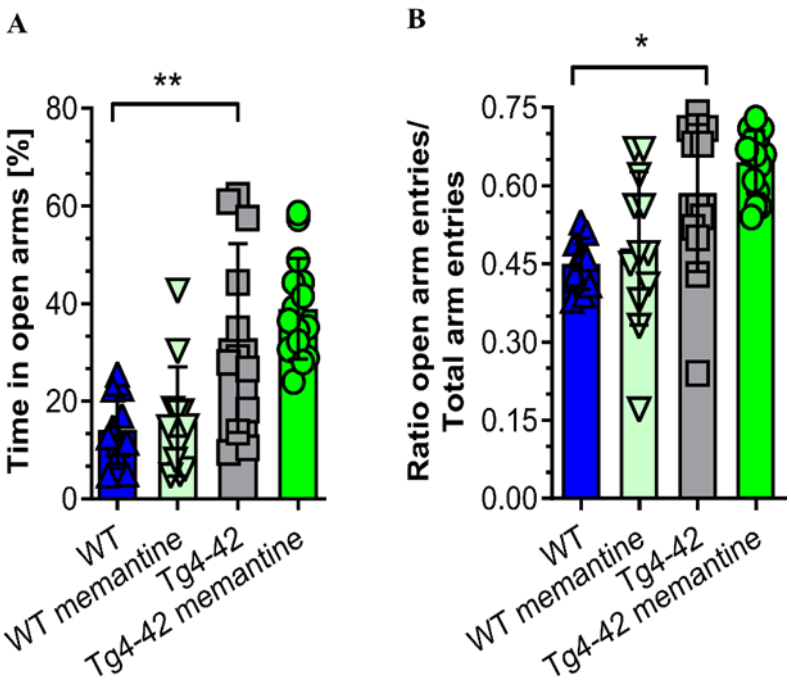

Figure 12: Chronic memantine treatment does not change the anxiety phenotype of Tg4-42^{hom} mice.
(A, B) The elevated plus maze test revealed that untreated Tg4- 42^{hom} mice show reduced anxiety levels, indicated by significantly increased time spent in open arms compared with WT animals. All data were given as mean ± SD. *$p < 0.05$, **$p < 0.01$. WT n = 12, WT memantine n = 14, Tg4-42^{hom} n = 12, and Tg4-42^{hom} memantine n = 14. Figure adapted from (Stazi and Wirths, 2021a).

3.1.2. Chronic memantine treatment partially ameliorates sensory-motor function in Tg4-42^{hom} mice

After four months of oral memantine treatment, motor performance of mice was evaluated with the rotarod and balance beam tasks (Figure 13). In the rotarod test, balance and motor skill learning and coordination are analyzed. While the WT and memantine-treated WT animals showed an improvement in their ability to stay on the rod over the two days of testing, aged-matched Tg4-42^{hom} display a significant impairment in motor skills as shown by overall reduced latencies to fall (Figure 13A, $p < 0.001$ vs WT). Memantine treatment did not rescue this motor deficit (Figure 13A). Moreover, Tg4-42^{hom} performed significantly worse than the aged-matched WT group in the balance beam task (Figure 13B, $p < 0.001$). This phenotype was ameliorated after four months of chronic memantine treatment, as shown by significantly increased latencies to fall from the beam in the memantine treated-Tg4-42^{hom} mice compared with the untreated group (Figure 13B, $p < 0.001$).

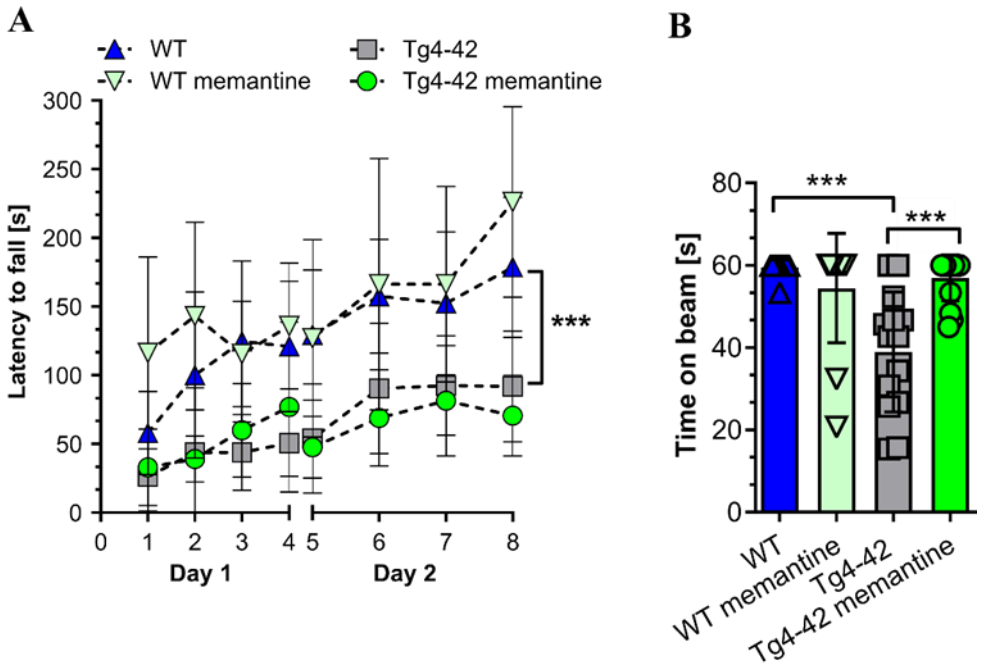

Figure 13: Memantine treatment has a partial effect on the motor performance of the Tg4-42^{hom} mice.
(A) The accelerating rotarod task revealed that Tg4-42^{hom} performed significantly worse compared to WT mice. No improvement was detected upon chronic memantine treatment in the Tg4-42^{hom} group. (B) Memantine treatment had a beneficial effect in the balance beam test, with the memantine-treated Tg4-42^{hom} mice performing at WT levels. All data were given as mean ± SD ***p <0.001. WT n = 12, WT memantine n = 14, Tg4-42^{hom} n = 12, and Tg4-42^{hom} memantine n = 14. Figure adapted from (Stazi and Wirths, 2021a).

3.1.3. Chronic memantine treatment restores recognition memory in Tg4-42^{hom} mice

Recognition memory was evaluated using the NOR task (Figure 14A). During the habituation day (open field, OF), no difference among all the groups with regard to the time spent in the in the centre of the arena, as well as speed or distance travelled could be detected (Figure 14B, C and D respectively). Twenty-four hours after the OF during the exploration phase, all groups spent an equal amount of time exploring each of the similar objects (Figure 14E). After another 24 hours during the testing phase, untreated Tg4-42^{hom} mice spent an equal amount of time exploring the familiar and novel object (Figure 14F), while untreated WT animals, as well as drug-treated WT and Tg4-42^{hom} mice spent significantly more time exploring the novel object (Figure 14F, $p < 0.001$). Calculation of the DI confirmed this observation, with untreated Tg4-42^{hom} mice showing a significantly lower DI than all other groups (Figure 14G, $p < 0.001$).

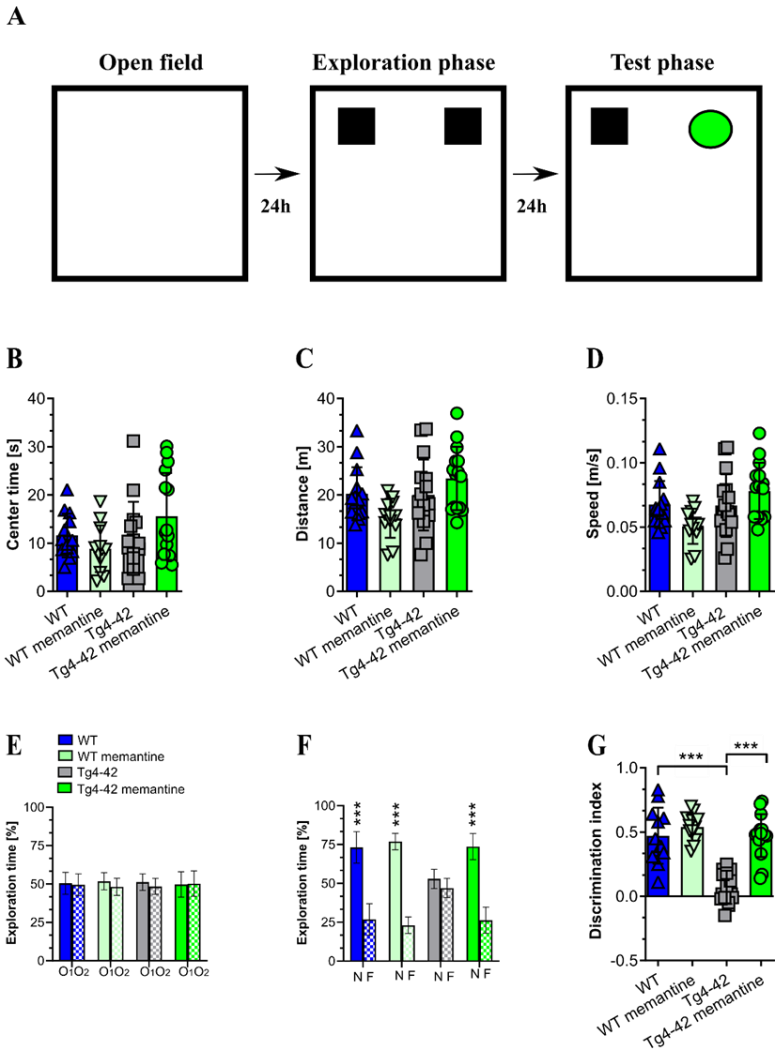

Figure 14: Chronic memantine treatment rescues impaired object recognition memory in Tg4-42^{hom} mice.
(A) Schematic drawing of the NOR test. (B) During the exploration phase, all groups spent ~ 50% of time exploring each of the two similar objects. (C) On the testing day, untreated Tg4-42^{hom} mice showed no object preference, while all other groups revealed a significant preference for the novel object. (D) Analysis of the DI indicates an inability of Tg4-42^{hom} mice to discriminate between objects, with significantly lower DI compared to all other groups. (O_1 = Object 1 O_2 = Object 2, N = Novel object, F = Familiar object). Two-way ANOVA followed by Bonferroni's post hoc test. All data were given as mean ± SD. ***$p < 0.001$. WT n = 12, WT memantine n = 14, Tg4-42^{hom} n = 12, and Tg4-42^{hom} memantine n = 14. Figure adapted from (Stazi and Wirths, 2021a).

3.1.4. Chronic memantine treatment prevents spatial reference memory deficits in Tg4-42^{hom} mice

Six-month-old Tg4-42^{hom} mice show severe spatial reference memory deficits (Hüttenrauch et al., 2016a). To analyse if long-term oral memantine treatment ameliorates these memory deficits, we carried out the MWM task. Both treated or untreated WT and Tg4-42^{hom} mice showed progressively decreased escape latencies over three days of cued training (Figure 15A), while no differences in swimming speeds were observed (Figure 15D). This initial phase of the MWM is essential to check if sensory (e.g., intact vision) or swimming deficits

bias the results and interpretation of the observed MWM data. During the following five days of acquisition training, all mice, regardless of treatment, displayed gradually decreased escape latencies and again no difference in swimming speed was detected (Figure 15B and E respectively). During the probe trial, carried out 24 h after the last acquisition trial, untreated Tg4-42^{hom} mice showed no significant preference for the target quadrant. This phenotype was rescued by chronic memantine treatment, as the memantine-treated Tg4-42^{hom} mice, spent significantly more time in the target quadrant compared to the average time spent in the other three pool quadrants (Figure 15C, $p < 0.05$ and $p < 0.001$ respectively). As no differences in swimming speeds were observed in the probe trial (Figure 15F), this is indicative of a rescue of spatial reference memory.

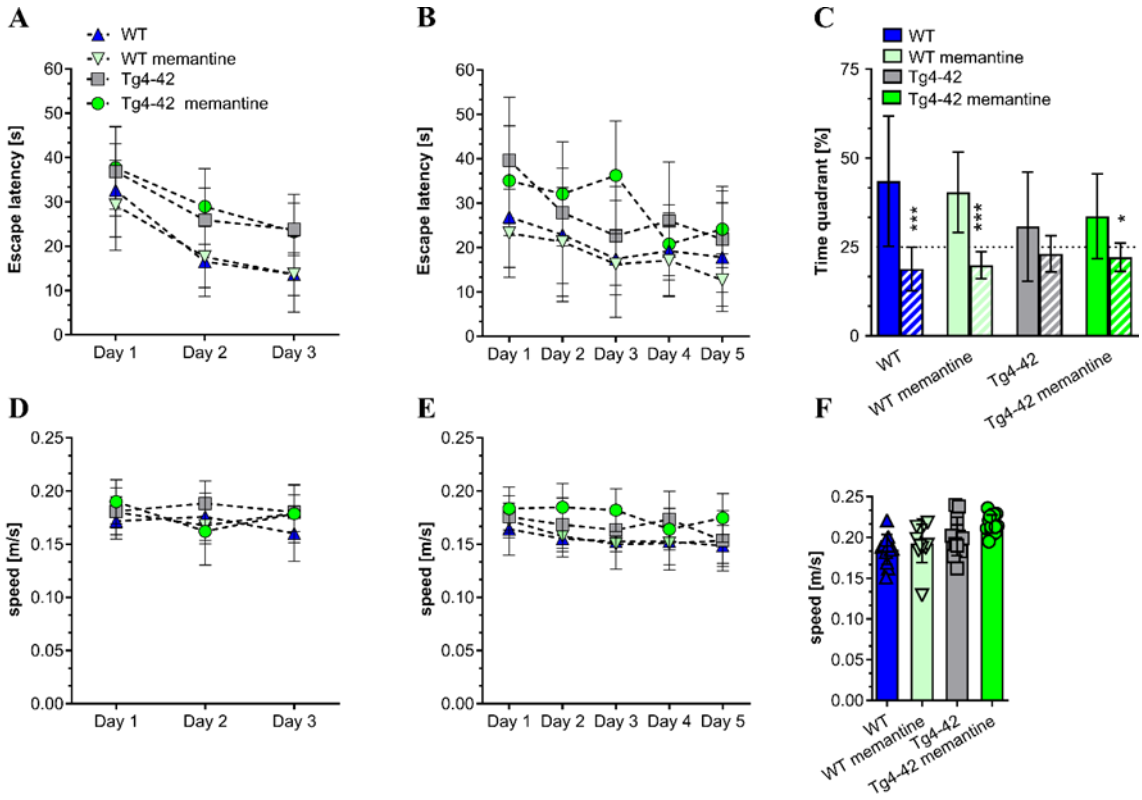

Figure 15: Memantine treatment rescues impaired spatial memory performance in Tg4-42^{hom} mice.
(A-B) All groups showed progressively reduced escape latencies during the cued and acquisition training. (C) During the probe trial, Tg4-42^{hom} showed no preference for any of the quadrants, while untreated WT, memantine-treated WT, and memantine-treated Tg4-42^{hom} mice spent significantly more time in the target quadrant (T) compared to all the other three quadrants (RLO), indicative of an intact spatial reference memory. (D-F) No differences in swimming speed were observed between all the groups in cued training, acquisition training, and probe trial. (A-E) Two-way repeated measures ANOVA followed by Bonferroni multiple comparisons test. (F) One-way ANOVA followed by Bonferroni's multiple comparisons test. *$p < 0.05$, ***$p < 0.001$. All data were given as mean ± SD. (T, target; L, left; R, right; O, opposite). WT n = 12, WT memantine n = 14, Tg4-42^{hom} n = 12, and Tg4-42^{hom} memantine n = 14. Figure adapted from (Stazi and Wirths, 2021a).

3.1.5. Chronic memantine treatment ameliorates hippocampal neuron loss in the CA1 area of Tg4-42^{hom} mice

As 6-month-old Tg4-42^{hom} mice present a drastic loss of CA1 pyramidal neurons (Hüttenrauch et al., 2016a), we examined whether chronic memantine treatment might exert neuroprotective effects. Therefore, we quantified the number of haematoxylin-stained neuronal nuclei in the hippocampal CA1 region in 6-month-old untreated and drug-treated WT and Tg4-42hom mice (Figure 16). An analysis discriminating between the proximal (extending to CA2) and the distal (towards subiculum) parts of the CA1 layer revealed no difference among treated and untreated WT mice. In contrast, a non-significant amelioration of ~ 4% in the proximal and a more pronounced ~ 17% amelioration of neuron loss in the distal CA1 part among Tg4-42^{hom} and Tg4-42^{hom} memantine-treated animals (Figure 16A and B respectfully, $p < 0.05$) was detected. Compared to the untreated WT control group, Tg4-42^{hom} mice displayed a neuron loss of ~ 37% in the proximal CA1 and ~ 57% in the distal CA1 (Figure 16A and B respectively), which is in good agreement with previous results showing a ~ 50% overall CA1 neuron loss compared to age-matched WT mice (Antonios et al., 2015). Interestingly, a correlation analysis between the discrimination index in the NOR and CA1 neuron numbers revealed no correlation for the proximal CA1 part (Figure 18A, Pearson r = 0.2719, $p = 0.3926$), but a highly significant correlation for the distal CA1 region (Figure 18B, Pearson r = 0.7189, $p = 0.0084$).

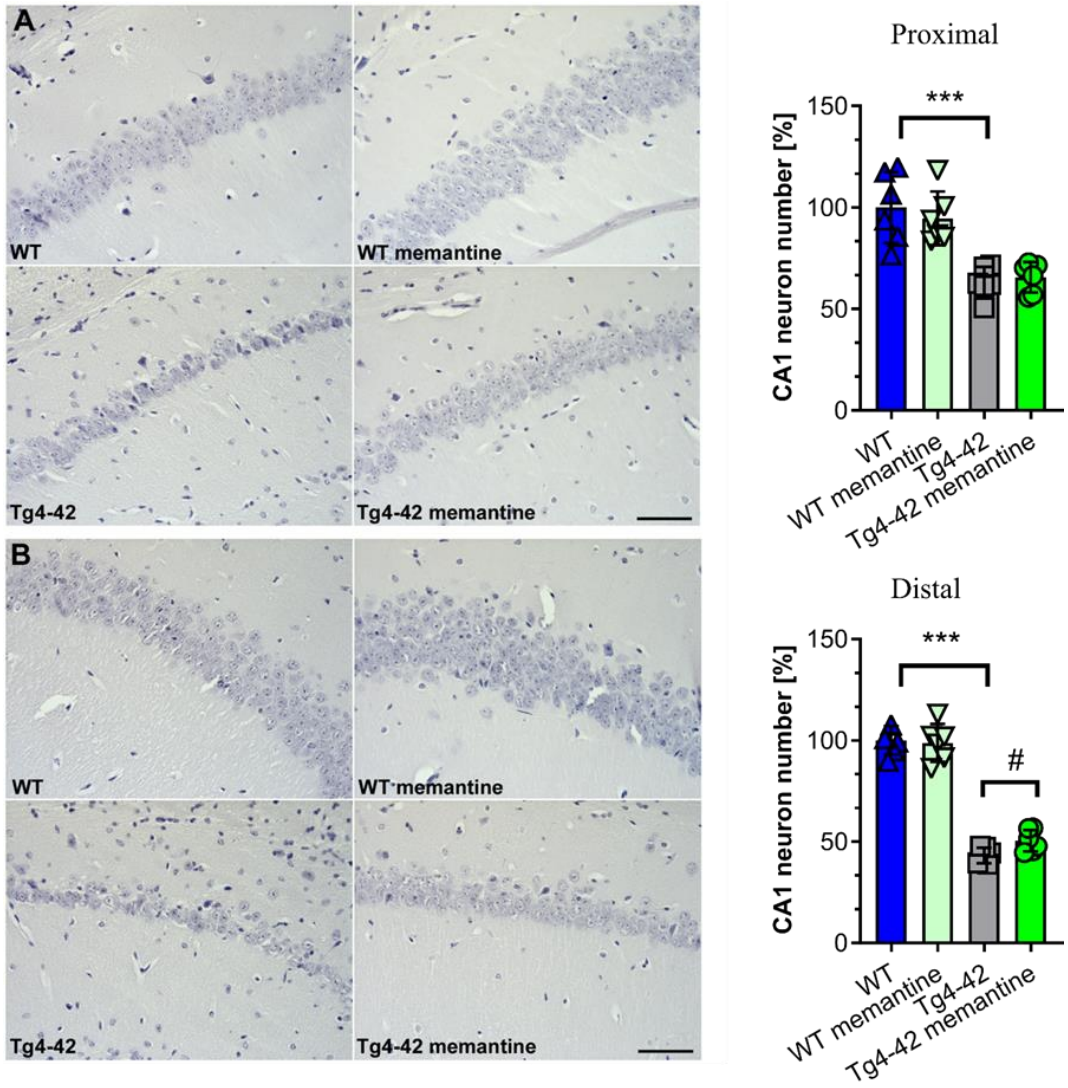

Figure 16: Memantine treatment decreases neuron loss in the CA1 region of the hippocampus of the Tg4-42^{hom} mice. (A) Analysis of haematoxylin-stained sections revealed significantly reduced proximal CA1 neuron numbers in Tg4-42^{hom} compared to WT mice. (B) This massive neuron loss was reduced in the distal CA1 upon drug treatment as Tg4-42^{hom} memantine mice showed significantly higher CA1 neuron numbers when compared to Tg4-42^{hom} untreated littermates. One-way ANOVA followed by the Mann–Whitney test. All data were given as mean ± SD. # $p < 0.05$, ***$p < 0.001$, scale bar: 50 µm. WT n = 6, WT memantine n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} memantine n = 6. Figure adapted from (Stazi and Wirths, 2021a).

3.1.6. Chronic memantine treatment ameliorates impaired DG neurogenesis

Compared with WT mice, Tg4-42^{hom} mice at 6 months of age showed a strongly reduced number of DCX-positive neurons in the DG of the hippocampus (Figure 17, $p < 0.001$). While chronic memantine treatment did not alter neurogenesis in WT mice, ~ 33% more DCX-positive neurons were detected in memantine-treated Tg4-42^{hom} mice compared with non-treated Tg4-42^{hom} littermates (Figure 17, $p < 0.05$). A correlation analysis revealed a

significant correlation between the neurogenesis rate and the performance in the NOR (Figure 18C, Pearson r = 0.6389, $p = 0.0253$)

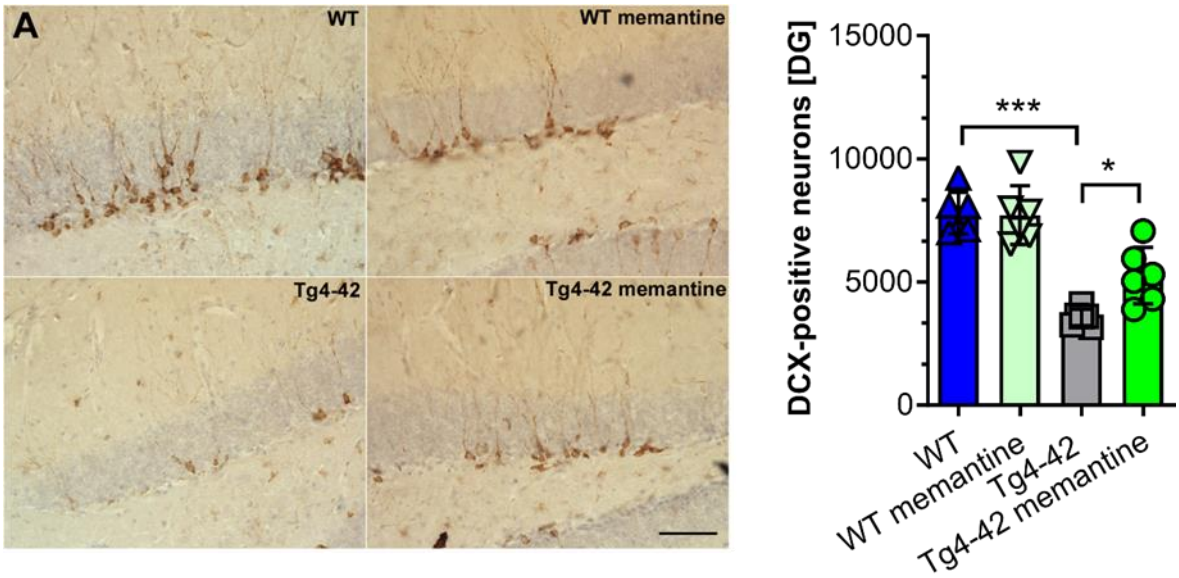

Figure 17: Chronic oral memantine treatment ameliorates impaired neurogenesis in the DG of Tg4-42^{hom} mice.
Analysis of doublecortin (DCX)-stained sections revealed a significantly reduced number of DCX-positive cells in Tg4-42^{hom} mice compared with untreated WT mice. While chronic memantine treatment did not alter the number of new-born cells in WT mice, a significantly increased number was detected in memantine-treated Tg4-42^{hom} mice. One-way ANOVA followed by Bonferroni's multiple comparisons test. *$p < 0.05$, ***$p < 0.001$. WT n = 6, WT memantine n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} memantine n = 6. Scale bar: 50 µm. Figure adapted from (Stazi and Wirths, 2021a).

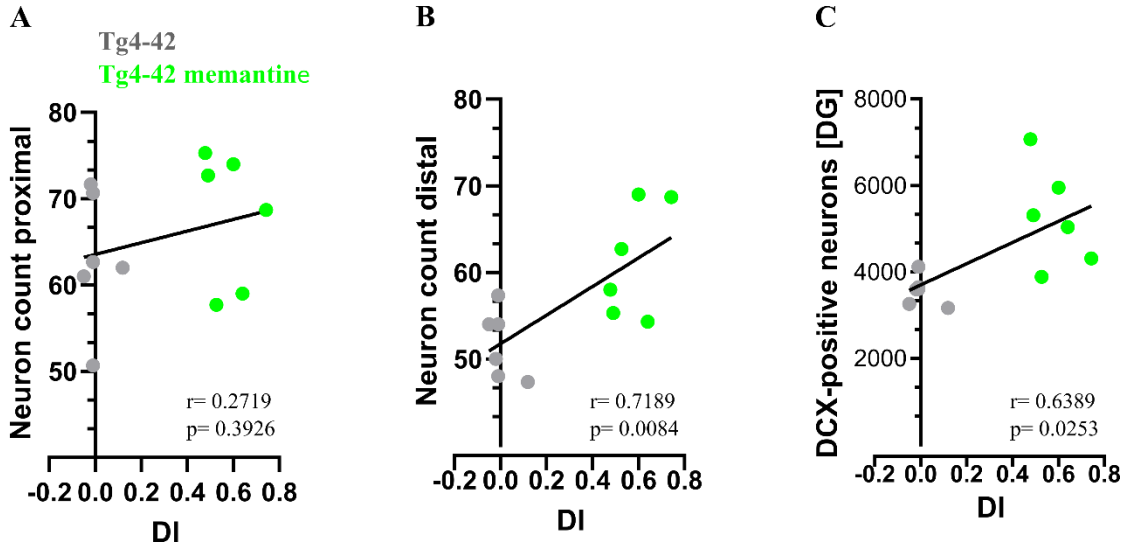

Figure 18: Correlation between behavioural performance and neuron count or DCX-positive cells after memantine treatment.
(A) While no significant correlation could be established between the DI in the NOR task and CA1 neuron number in the proximal part (Pearson r = 0.2719, $p = 0.3926$), (B) a highly significant correlation was detected between DI and the distal CA1 neuron number (Pearson r = 0.7189, $p = 0.0084$). (C) The same holds true for the correlation between neurogenesis rate expressed by the number of DCX-positive neurons in the dentate gyrus (DG) and DI (Pearson r = 0.6389, $p = 0.0253$). Tg4-42^{hom} n = 6 and Tg4-42^{hom} memantine n = 6. Figure adapted from (Stazi and Wirths, 2021a).

3.2. PROJECT II: The effect of long-term oral caffeine intake on the pathology of Tg4-42^{hom} and 5xFAD mice

Two-month-old WT, Tg4-42^{hom} and 5xFAD mice were treated for four months with caffeine (300 mg/L) in drinking water (Figure 19A). At six months of age at the end of the treatment, mice were subjected to a battery of behavioural tests with ongoing treatment. At the end of the behavioural testing phase, animals were sacrificed, and tissues were collected for further biochemical and/or stereological analysis. To assess if the caffeine has an impact on the physiological status of the animals, their body weight together with liquid consumption was monitoring throughout the behavioural test period. No differences in water consumption (Figure 19B) or body weight (Figure 19C) were found among all groups irrespective of the treatment or genotype.

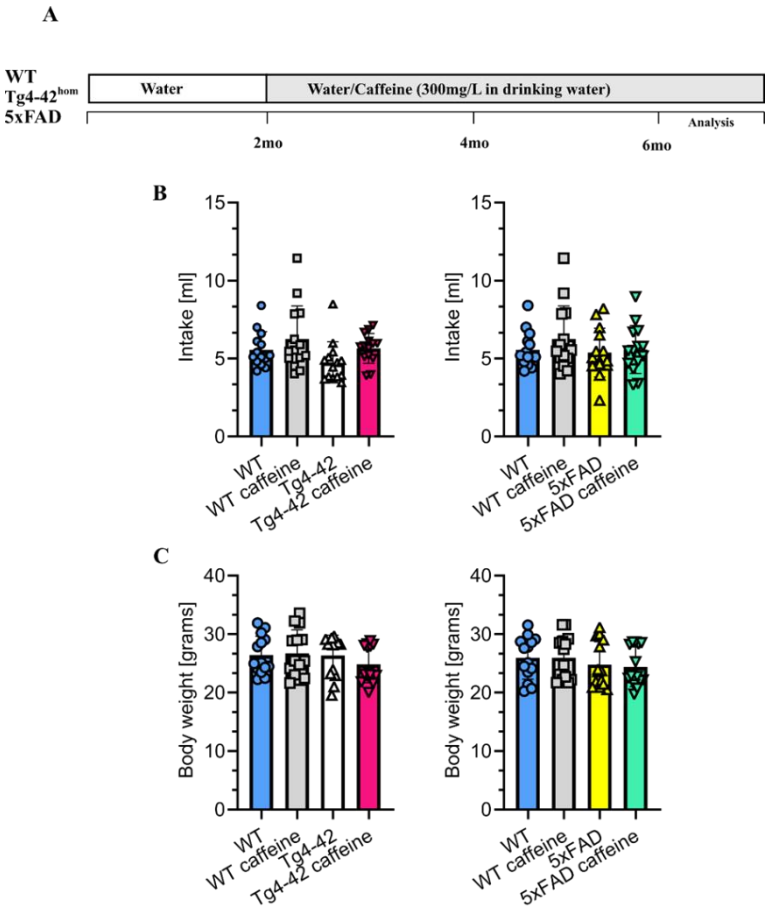

Figure 19: Experimental design and physiological status of mice after caffeine treatment.
(A) Schematic illustration of the experimental design. (B) Caffeine treatment had no effect on the body weight or (C) water consumption among the different groups. One way ANOVA followed by Bonferroni multiple comparisons tests. WT n = 14, WT caffeine n = 14, Tg4-42^{hom} n = 14, Tg4-42^{hom} caffeine n = 14, 5xFAD n = 14, and 5xFAD caffeine n = 13. Data are presented as mean ± SD.

3.2.1. Part I: Caffeine treatment delays hippocampal neurodegeneration and rescues memory deficits in the Tg4-42^{hom} mouse model of AD

3.2.1.1. Limited effect of caffeine on the motor performance of the Tg4-42^{hom}

After four months of chronic caffeine treatment, motor performance of the animals was analysed using the rotarod and the balance beam tasks. Six-month-old Tg4-42^{hom} mice display motor deficits (Wagner et al., 2019) that were partially ameliorated after the caffeine treatment. In fact, at 6 months of age, Tg4-42^{hom} showed a worsened performance in the rotarod test when compared to WT littermates (Figure 20A). This phenotype could not be ameliorated upon caffeine treatment, as no difference was detected in the rotarod task between untreated and the caffeine-treated Tg4-42^{hom} group (Figure 20A). Moreover, Tg4-42^{hom} performed significantly worse than age-matched WT mice in the balance beam task (Figure 20B, $p < 0.001$). Caffeine treatment completely reversed this phenotype was in Tg4-42^{hom} mice as shown by overall higher latencies to stay on the beam or reaching the escape platform (Figure 20B, $p < 0.001$).

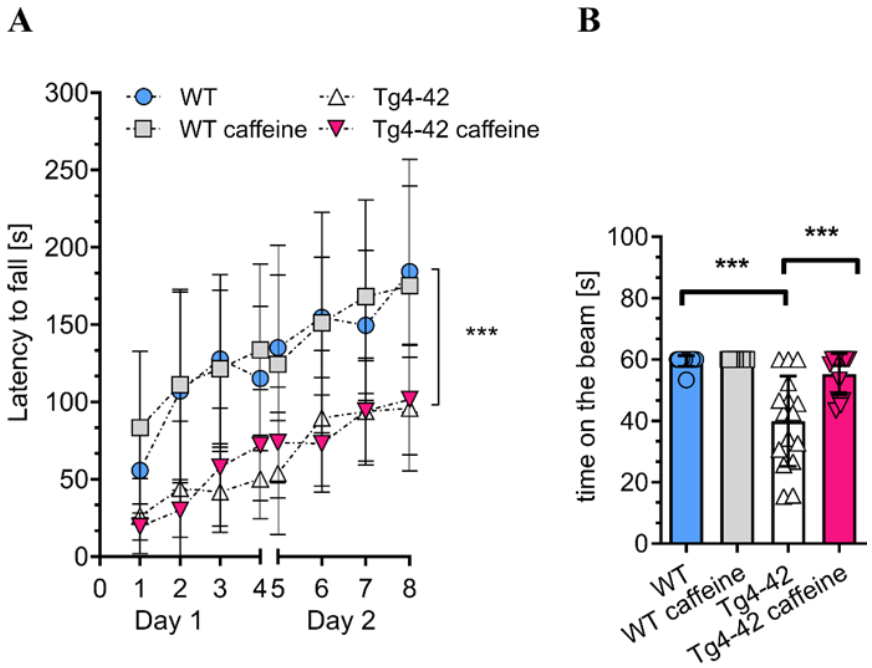

Figure 20: Caffeine treatment had limited effects on the sensory-motor performance of Tg4-42^{hom} mice.
(A) The rotarod test showed no improvement in caffeine-treated Tg4-42^{hom} mice compared to control mice receiving vehicle. (B) The balance beam task revealed a rescue of the motor phenotype, as Tg4-42^{hom} mice stayed significantly longer on the beam compared to untreated mice. One way ANOVA followed by Bonferroni multiple comparisons test ***$p < 0.001$. WT n = 14, WT caffeine n = 14, Tg4-42^{hom} n = 14, and Tg4-42^{hom} caffeine n = 14. Data are presented as mean ± SD.

3.2.1.2. Long-term oral caffeine intake rescues both recognition and spatial memory deficits in Tg4-42^{hom}

The OF represents the habituation phase for the NOR task (Figure 21), which was used to analyse recognition memory and novelty preference in Tg4-42^{hom}. No obvious differences among the genotypes or treatment were detected in this task with regard to time spent in the centre of the arena or distance travelled (Figure 21B and C). Twenty-four hours later, on the first day of the NOR (exploration day), all groups spent an equal amount of time exploring the two identical objects (Figure 21D). When tested for recognition memory 24 h later, Tg4-42^{hom} mice did not show a preference for any of the objects, indicating a deficit in recognition memory (Figure 21D). Untreated WT as well as caffeine-treated WT and Tg4-42^{hom} mice explored the novel object (N) respect the familiar one (F) for a significantly longer time period, indicating intact object recognition memory (Figure 21D, $p < 0.001$). A calculation of the discrimination index (DI) confirmed these results, showing that Tg4-42^{hom} caffeine mice had significantly higher discrimination indices compared to the untreated Tg4-42^{hom} group (Figure 21F, $p < 0.001$).

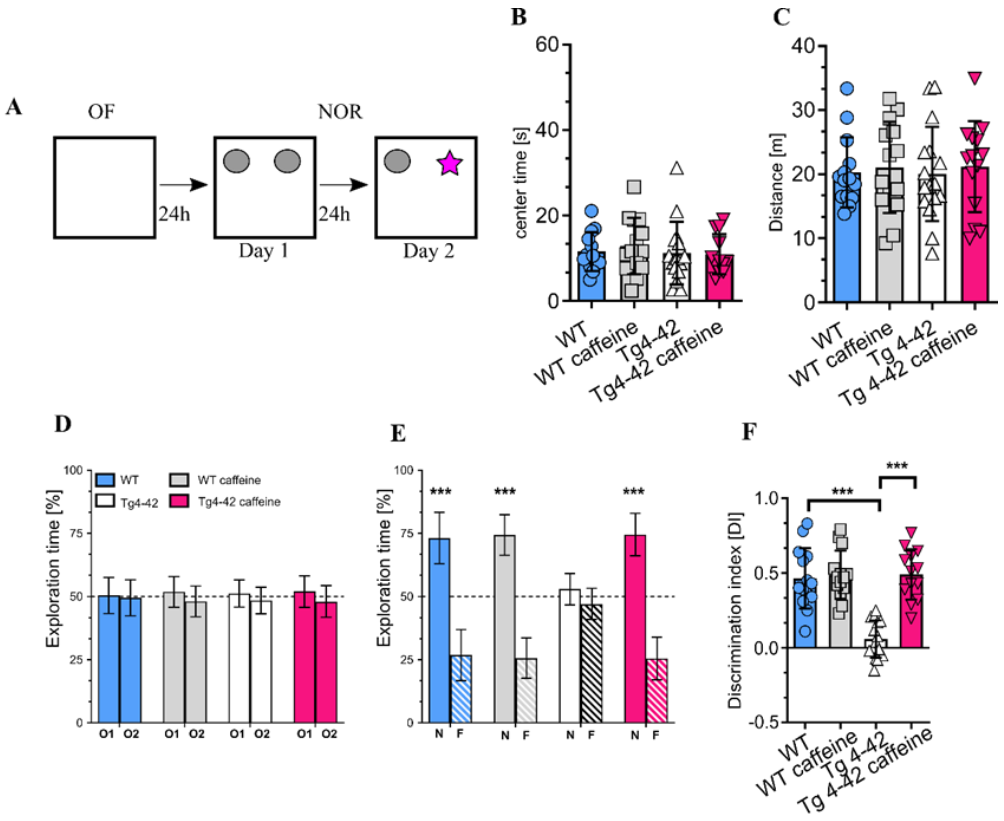

Figure 21: The effect of caffeine treatment on recognition memory performance.
(A) The NOR task was used to test recognition memory. (B) In the OF, no significant difference between vehicle and caffeine-treated groups with regard to the time spent in the centre and (C) distance travelled were detected. (D) During the exploration phase on day 1, treated or untreated WT and Tg4-42^{hom} mice spent an equal amount of time with each of the identical objects 1 and 2 (O1, O2). (E) During the test trial on day 2, WT as well as caffeine-treated WT and Tg4-42^{hom} mice spent significantly more time with the novel object (N) while untreated Tg4-42^{hom} mice did not show a difference between the time spent with the familiar (F) and novel object (N). (F) Recognition memory was also evaluated using the DI, showing that WT and drug-treated groups have a significant higher score compared to untreated Tg4-42 mice. (B, C, F) One-way ANOVA followed by Bonferroni multiple comparisons test. (D, E) Two-way ANOVA followed by Bonferroni multiple comparisons test. *** $p < 0.001$. WT n = 14, WT caffeine n = 14, Tg4-42^{hom} n = 14, and Tg4-42^{hom} caffeine n = 14. Data are presented as mean ± SD.

At 6 months of age, Tg4-42^{hom} mice present with serious spatial reference memory deficits (Hüttenrauch et al., 2016a). To analyse whether long-term caffeine treatment results in an amelioration of these behavioural deficits, the Morris water maze (MWM) test was performed. All investigated groups showed decreased escape latencies over the three days of cued training and the five days of the acquisition phase (Figure 22A and B). However, the untreated WT showed shorter escape latencies compared to Tg4-42^{hom} during the cued phase (Figure 22A and B, $p < 0.01$), while caffeine-treated WT mice showed reduced latencies in comparison to untreated WT mice in the acquisition phase (Figure 22B, $p < 0.05$). During the initial cued phase, the Tg4-42^{hom} swam significantly slower compared to the WT control

group (Figure 22C, $p < 0.05$), however, all groups displayed comparable swimming speeds during the acquisition training period (Figure 22D). In the probe trial, Tg4-42^{hom} mice showed no clear preference for the target quadrant (Figure 22E). In contrast, caffeine-treated mice showed a preservation of spatial reference memory as they spent significantly more time in the target quadrant (T) compared to average of the other quadrant (RLO), with a performance at WT levels (Figure 22C, $p < 0.001$ vs RLO). Again, no differences in the swimming speed were observed in all the groups during the final probe trial (Figure 22F). Representative occupancy plots of all groups revealed that WT control and caffeine treated mice (WT and Tg4-42^{hom}) focused their search on the initial platform location during the probe trial, while untreated transgenic animals showed a more random searching strategy (Figure 22G).

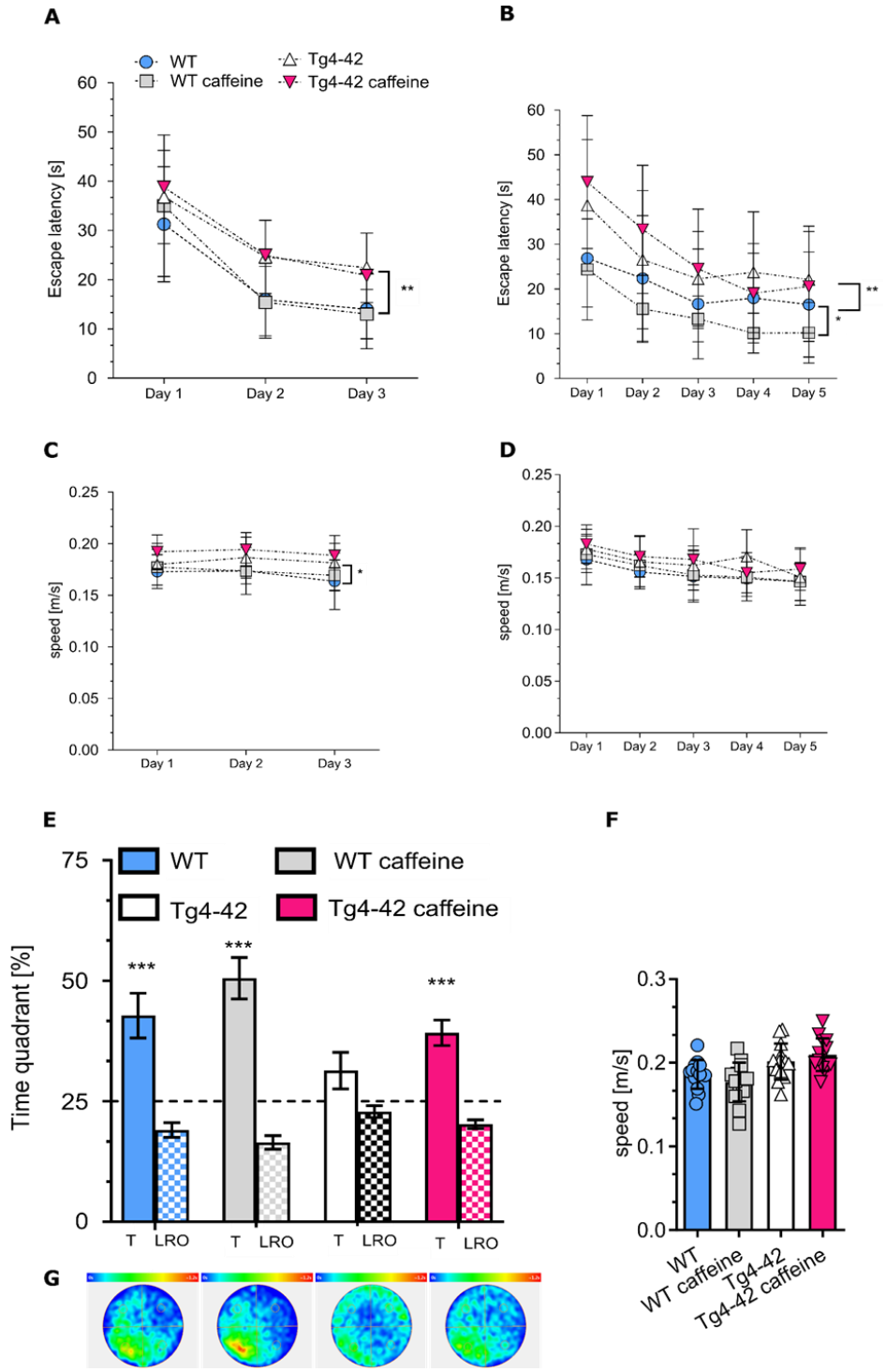

Figure 22: Impaired spatial reference memory in Tg4-42^{hom} mice is rescued upon caffeine treatment.
(A) Untreated and caffeine-treated WT and Tg4-42^{hom} mice showed decreased escape latencies over the three days of cued training. However, Tg4-42^{hom} mice showed a significantly poorer performance than WT mice as shown by overall higher escape latencies during the entire duration of the cued training. (B) Similar to A, mice of all groups showed progressively reduced escape latencies over the five days of acquisition training. Caffeine-treated WT mice displayed an improved spatial learning performance compared to the control group, as seen by lower escape latencies over the entire acquisition-training period. (C) Moreover, Tg4-42^{hom} presented a significantly reduced swimming speed respect the WT control group. (D) No differences in swimming speed were observed among all the groups in the acquisition training. (E) Tg4-42^{hom} mice showed no preference for any of the quadrants during the probe trial. WT control as well as caffeine-treated WT and Tg4-42^{hom} mice displayed an intact spatial reference memory as they spent significantly more time in the target quadrant (T) compared to all the other quadrants (LRO). (F) Again, no difference in the swimming speed were find during the probe trial. (G) The occupancy plots indicate exemplarily the averaged swimming traces of untreated and treated animals during the probe trial. (A-E) Two-way ANOVA followed by Bonferroni multiple comparison test. (F) One-way ANOVA followed by Bonferroni multiple comparisons test. * p <0.05; ** p < 0.01; *** p < 0.001. WT n=14, WT caffeine n=14, Tg4-42^{hom} n=14, and Tg4-42^{hom} caffeine n=14. All data were given as mean ± SD.

3.2.1.3. Caffeine ameliorates hippocampal neuronal loss in the CA1 area of Tg4-42^{hom} mice

To analyse if a chronic oral caffeine intake positively influences CA1 neuron numbers of Tg4-42^{hom} mice, we quantified the number of haematoxylin-stained neuronal nuclei in a defined area of the CA1 region of the hippocampus in 6-month-old untreated and drug-treated WT and Tg4-42^{hom} mice. A detailed analysis, discriminating between the distal (towards subiculum Figure 23A) and the proximal part of the CA1 area (extending to CA2, Figure 23D), revealed that Tg4-42^{hom} mice displayed a neuron loss of 58% in the distal CA1 and ~41% in the proximal CA1 area compared to the untreated WT control group (Figure 23B and D respectively). This is in good agreement with previous results showing an ~50% overall CA1 neuron loss compared to age-matched WT mice (Antonios et al., 2015). No difference between caffeine-treated and untreated WT mice could be detect, while, in comparison to the untreated-WT group, caffeine-treated Tg4-42^{hom} animal displayed a neuron loss of ~43% (distal portion), significantly different from the untreated Tg4-42^{hom} group and a non-significant neuron loss of ~27 in the proximal portion (Figure 23B and D, $p < 0.05$).

A correlation analysis between the discrimination index in the NOR and CA1 neuron numbers revealed a highly significant correlation for the distal CA1 area (Figure 25A and C, Pearson r = 0.8020, $p = 0.0030$), but no correlation for the proximal CA1 part (Figure 25B, Pearson r = 0.5932, $p = 0.0544$).

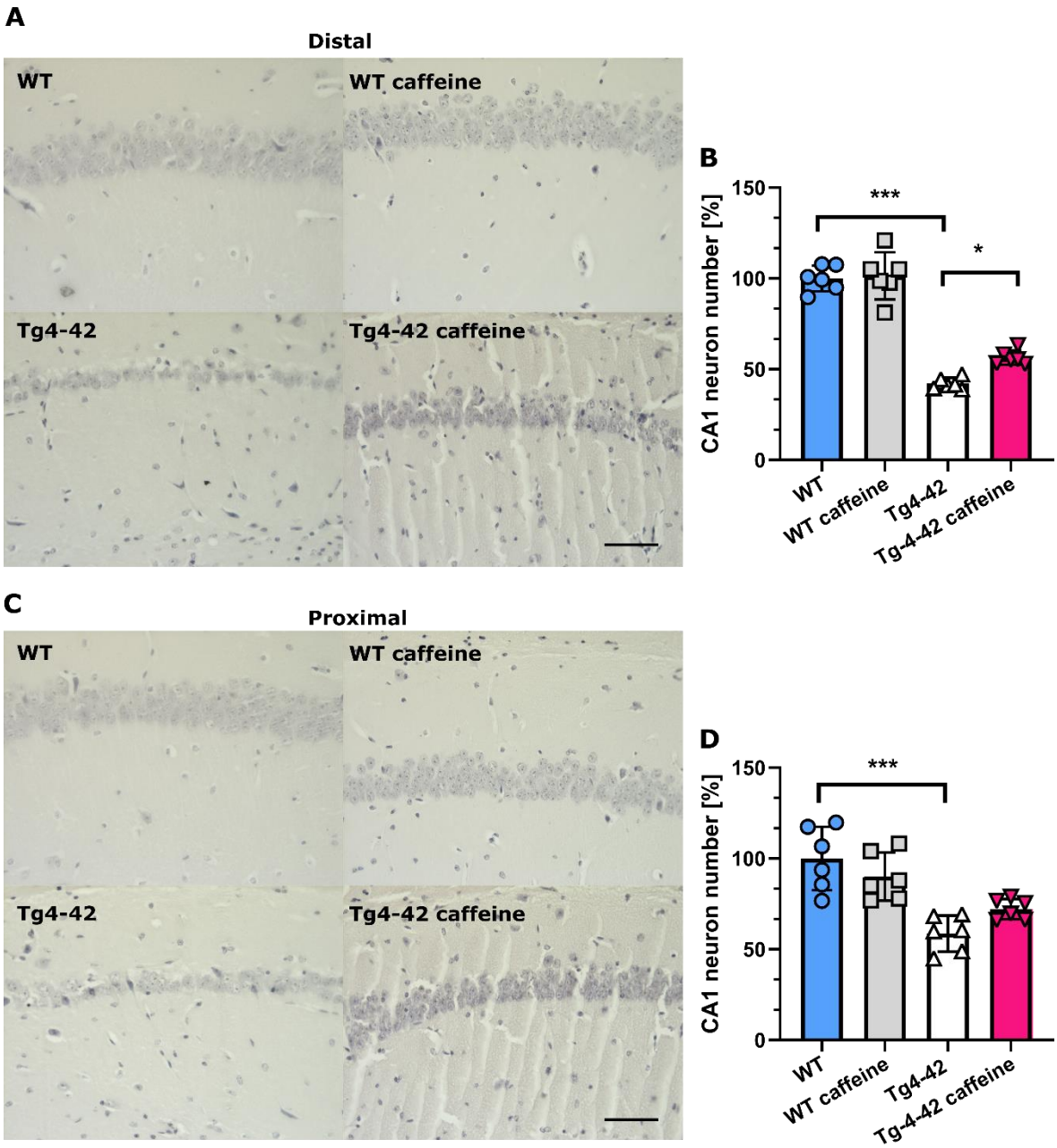

Figure 23: Caffeine treatment decreases the hippocampal neuronal loss in the distal portion of the CA1 area in the Tg4-42^{hom} mice.
(A, C) Representative images of the distal and proximal portion of the CA1 area of the hippocampus of all the groups. (B, D) Analysis of haematoxylin-stained sections revealed significantly reduced CA1 neuron numbers in the distal (-~58%) and proximal portion in Tg4-42^{hom} mice compared to WT. While no difference between caffeine-treated and control WT mice could be detect, a significant amelioration of neuron loss after caffeine treatment was noted in distal area (-~43%) as well as a non-significant amelioration in the proximal CA1 part in Tg4-42^{hom} caffeine-treated compared to untreated animals. (B, D) Two-way ANOVA followed by Bonferroni multiple comparison tests. (C, F). *$p < 0.05$; ***$p < 0.001$. WT n = 6, WT caffeine n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} caffeine n = 6. All data were given as mean ± SD. Scale bars = 50 µm.

3.2.1.4. Caffeine ameliorates impaired DG neurogenesis of the Tg4-42^{hom}

Stereological analysis of DCX-positive cells in the DG revealed a significant and strongly reduced number of DCX-positive neurons in 6-month-old Tg4-42^{hom} mice compared to the

WT mice (Figure 24, $p < 0.001$), as already shown in Figure 17. Interestingly, long-term oral caffeine treatment increased the numbers of DCX-positive cell in both WT and Tg4-42 caffeine-treated mice compared with the untreated littermates (Figure 24, $p < 0.001$). A correlation analysis revealed a significant correlation between the neurogenesis rate and the performance in the NOR (Figure 25C, Pearson $r = 0.9309$, $p = 0.001$).

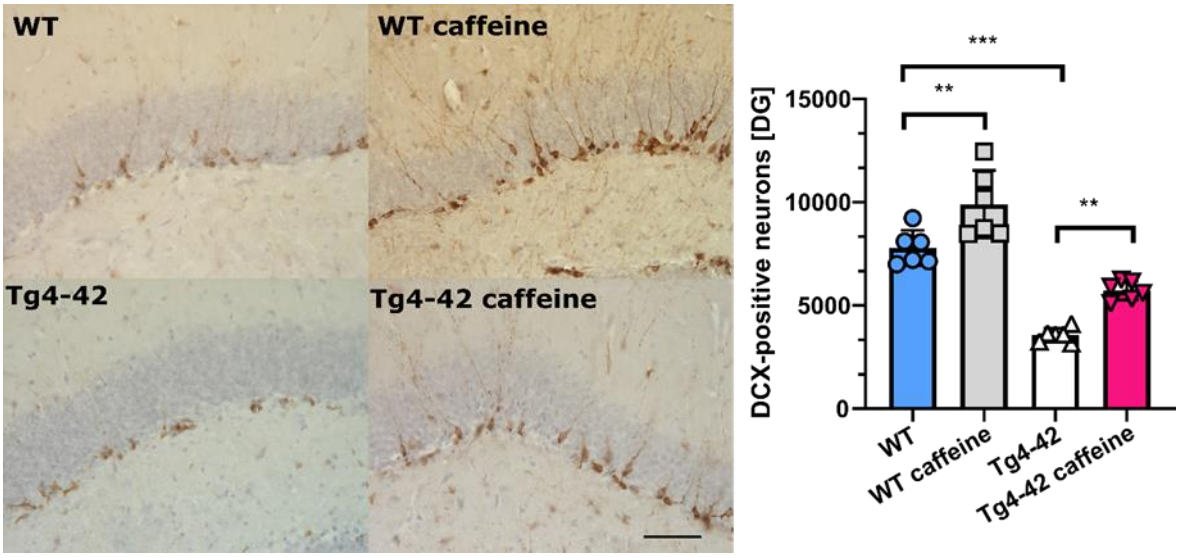

Figure 24: Caffeine treatment increases neurogenesis in the WT and Tg4-42^{hom} mice.
Analysis of doublecortin (DCX)-stained sections revealed a significantly reduced number of DCX-positive cells in Tg4-42^{hom} mice compared to untreated WT mice. Chronic oral caffeine intake altered the number of new-born neurons in both WT and Tg-4-42^{hom} caffeine mice compared to the respective vehicle control group. Two-way ANOVA followed by Bonferroni's multiple comparison test. ** p <0.001; ***p < 0.001. WT n = 6, WT caffeine n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} caffeine n = 6. All data are given as mean ± SD. Scale bar: 50µm.

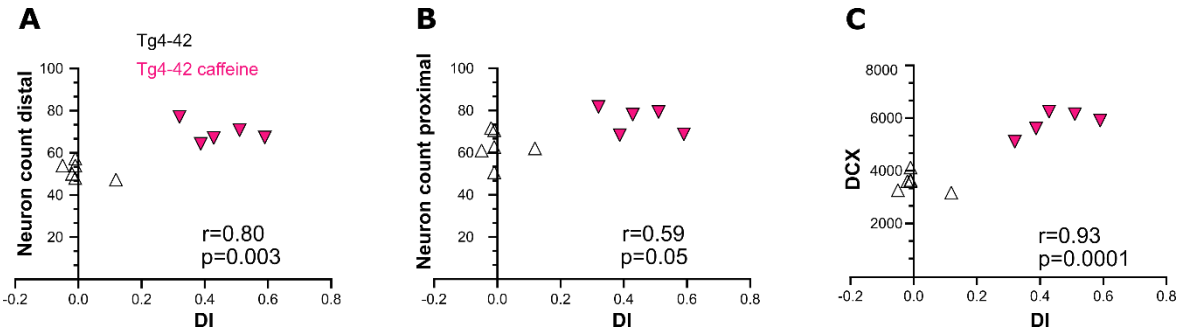

Figure 25: Correlation between behavioural performance and neuron count and DCX positive cells count after caffeine treatment.
(A) A highly significant correlation was detected between Discrimination index (DI) in the NOR and the neuron number in the distal CA1 (Pearson r = 0.80, p = 0.003), (B) while no significant correlation could be established between the DI and CA1 neuron number in the proximal part (Pearson r = 0.5932, p = 0.05) (C). A highly significant correlation could be established between neurogenesis rate expressed by the number of DCX-positive neurons in the dentate gyrus (DG) and DI (Pearson r = 0.93, p = 0.0001). WT n = 6, WT caffeine n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} caffeine n = 6.

3.2.2. Part II: Chronic oral caffeine treatment has limited effects on the pathology of 5xFAD mice

3.2.2.1. Caffeine rescues spatial memory deficits 5xFAD mice

5xFAD transgenic mice display spatial memory deficits in hippocampus-related tasks, such as novel object location (De Pins et al., 2019). In the novel object location test (NOL), spatial long-term memory was evaluated. The OF represents the habituation phase for the NOL task (Figure 26A) and no obvious differences among the genotypes or treatment were detected with regard to time spent in the centre of the arena or distance travelled in this task (Figure 26B and C). Twenty-four hours after the OF, mice were trained in the presence of two indistinguishable objects (O_1 and O_2), and no difference in percentage of exploration time could be found between the groups (Figure 26D). Another 24 h later, when tested for spatial memory assessed as the percentage of time exploring the object placed in a new location (NL) versus the time exploring the object placed in the familiar location (FL), untreated 5xFAD mice exhibited a significantly lower preference for the displaced object compared to the caffeine-treated group (Figure 26E, $p < 0.001$). A calculation of the discrimination index (DI) confirmed this finding as 5xFAD mice treated with caffeine showed significantly higher discrimination indices compared to their untreated littermates (Figure 26F, $p < 0.001$).

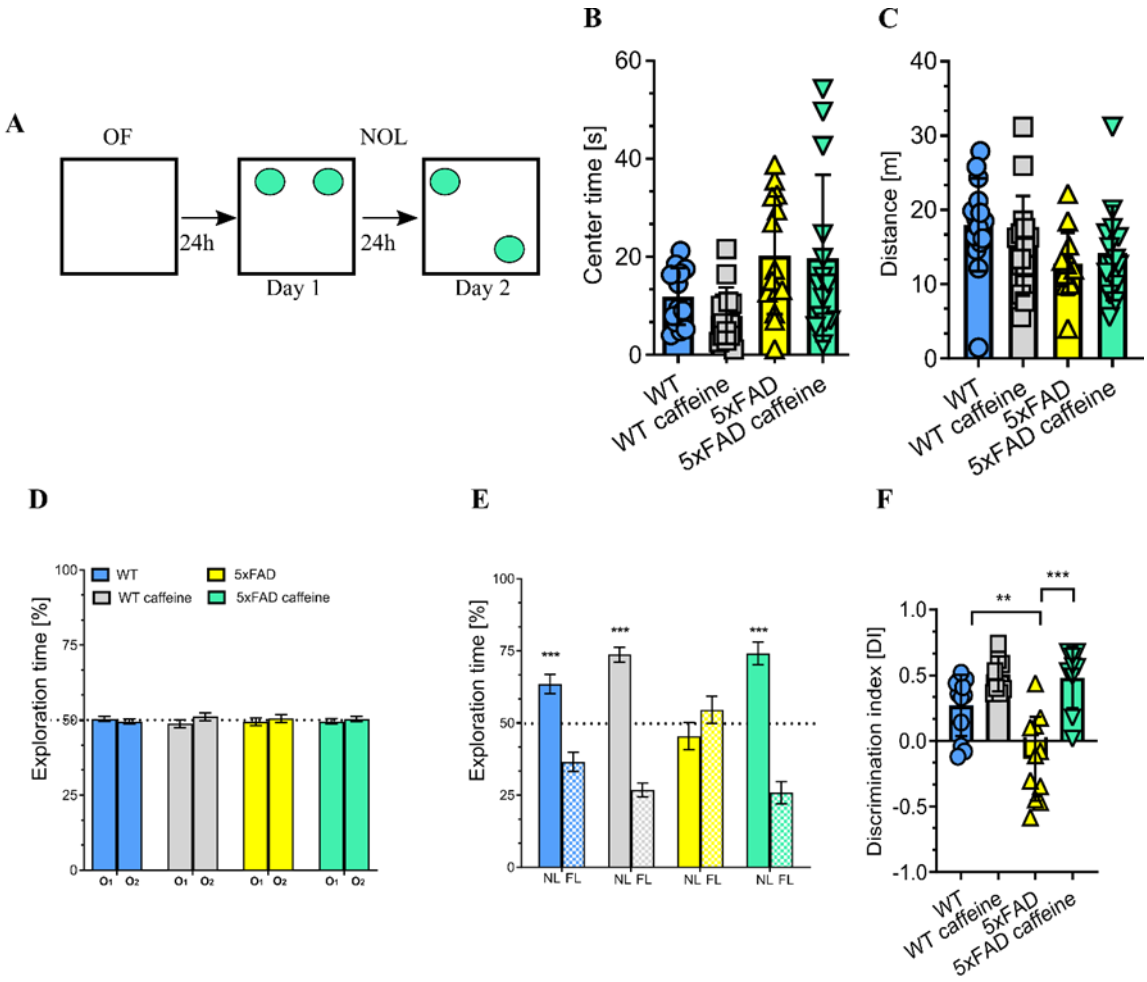

Figure 26: Impaired spatial reference memory in 5xFAD mice is rescued upon long-term oral caffeine treatment. (A) Schematic representation of the NOL task. (B, C) Effect of prolonged caffeine treatment on locomotors activity and exploration behaviour in 5xFAD mice. No significant difference between vehicle and caffeine-treated groups with regard to the time spent in the centre and distance travelled could be detected. (D) During the training phase on day one, all experimental groups spent an equal amount of time exploring each of the similar objects (O1, O2). (E) During the testing phase 24 h later, untreated 5xFAD mice did not show a preference of any of the object. In contrast, caffeine-treated transgenic mice spent significantly more time with the object placed in the new location (NL) compared with the familiar one (FL). (F) Spatial memory was also evaluated by calculating the DI and treated 5xFAD mice showed a significantly higher score than the untreated group. (D, E) Two-way ANOVA followed by Bonferroni multiple comparisons test. (B, C, F) One-way ANOVA followed by Bonferroni multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$. WT n = 14, WT caffeine n = 14, 5xFAD n = 14, and 5xFAD caffeine n = 13. Data are presented as mean ± SD.

3.2.2.2. Caffeine does not influence amyloid deposition and inflammatory status in brains of 5xFAD mice

5xFAD mice show abundant extracellular amyloid deposition starting at 2 months of age in the hippocampus, cortex, and thalamus (Oakley et al., 2006; Richard et al., 2015). In order to evaluate if chronic oral caffeine treatment was able to ameliorate amyloid pathology, a plaque load analysis was performed in untreated and caffeine-treated 5xFAD mice. Sagittal brain sections of 6-month-old 5xFAD and 5xFAD caffeine mice were stained with different

Aβ antibodies (24311 pan-Aβ and 82E1 Aβ1-x). Using 24311 and 82E1 no difference could be detected among the two groups in all areas analysed (Figure 27A and D).

At two months of age, together with the amyloid deposits, neuroinflammatory changes become obvious in 5xFAD mice (Oakley et al., 2006). To establish whether caffeine consumption ameliorates neuroinflammation, immunohistochemical staining and quantifications with the reactive astrocyte marker GFAP were done. No differences in GFAP signal could be detected in cortex, dentate gyrus and thalamus between 6-month-old untreated and caffeine-treated 5xFAD mice (Figure 27E and F).

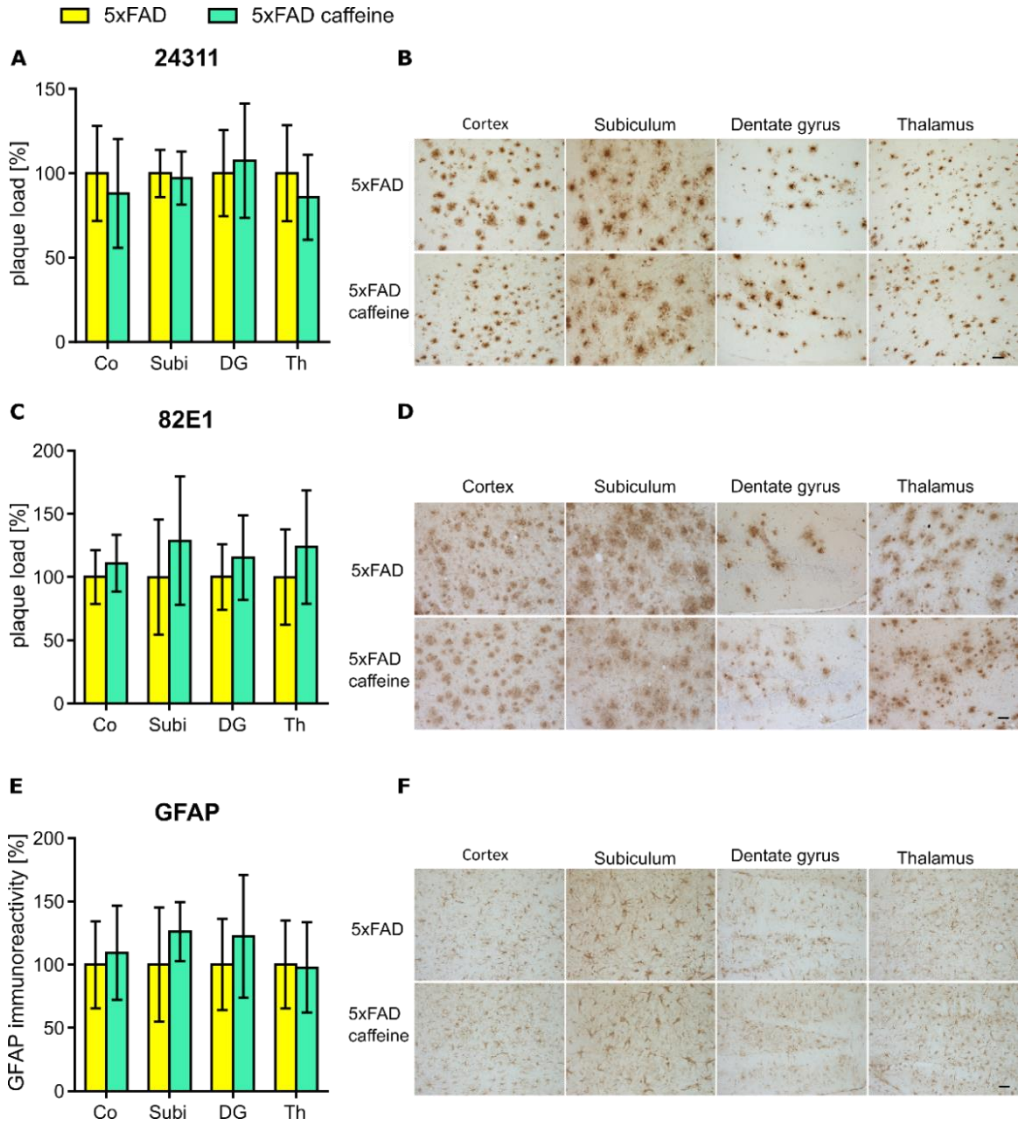

Figure 27: Quantification of Aβ plaque load and neuroinflammation in vehicle- and caffeine-treated 5xFAD mice. (A-D) Plaque load quantification in cortex (Co), subiculum (Subi), dentate gyrus (DG) and thalamus (Th) with a pan-Aβ (24311) and an N-terminal specific antibody (82E1) showed that caffeine treatment had no effect on Aβ plaque deposition in any of the regions analysed. (E-F) Using an antibody against the astrocytic marker GFAP, both groups showed comparable astrocytosis in all four brain regions analysed. (A, C, E) Two-way ANOVA followed by Bonferroni multiple comparisons test. 5xFAD n = 11 and 5xFAD caffeine n = 12. Data are given as mean ± SD. Scale bar =100 µm.

As previously shown, $A\beta_{1–42}$ is one of the most abundant Aβ peptide present in the brains of 5xFAD mice (Wittnam et al., 2012). To analyse whether caffeine treatment has an influence on the levels of this peptide, $A\beta_{1–42}$ was quantified using ELISA in SDS-soluble protein fraction from cortex and hippocampus. No differences in $A\beta_{1–42}$ levels could be detected between the groups (Figure 28). (ELISA measurements were performed by Sandra Lehmann, Department of Neuropathology, Heinrich Heine University, Düsseldorf).

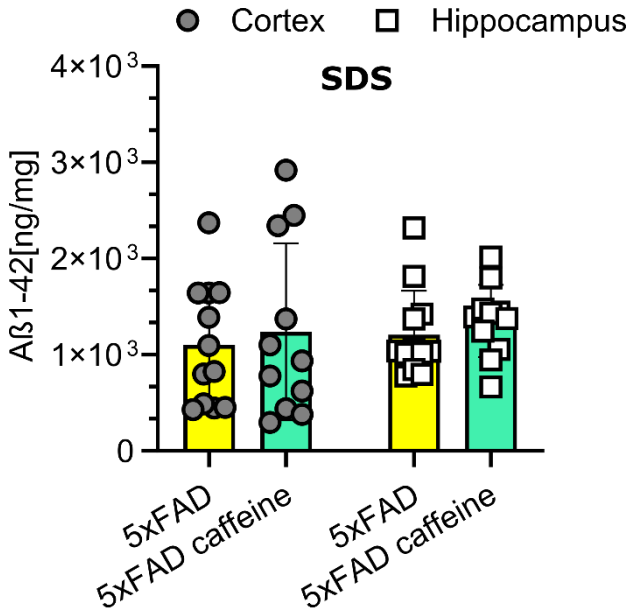

Figure 28: ELISA quantification of $A\beta_{1–42}$ in cortex and hippocampus.
No differences in SDS fractions in either cortex and hippocampus area in untreated or caffeine-treated 5xFAD mice were observed in $A\beta_{1-42}$ levels. 5xFAD n = 12 and 5xFAD caffeine n = 11. All data were given as mean ± SD.

3.2.2.3. Caffeine does not influence APP processing in brains of 5xFAD mice

In order to evaluate whether long oral assumption has an effect on APP processing *in vivo*, SDS-Page and Western blots were performed. APP processing in the hippocampus of 5xFAD caffeine mice was compared to untreated control animals. APP C-terminal fragments (CTFs) were detected in the SDS-soluble fraction (Figure 29A) and total secreted APP (APPs) and sAPP-α were detected in the TBS-soluble fraction (Figure 29B and C respectively). Levels of APP CTFs, APPs total and sAPP-α remained unchanged upon caffeine treatment in 5xFAD mice, leading to the suggestion that caffeine treatment does not seem to affect APP processing in the current experimental setting. (WB analysis were

performed by Sandra Lehmann, Department of Neuropathology, Heinrich Heine University, Düsseldorf).

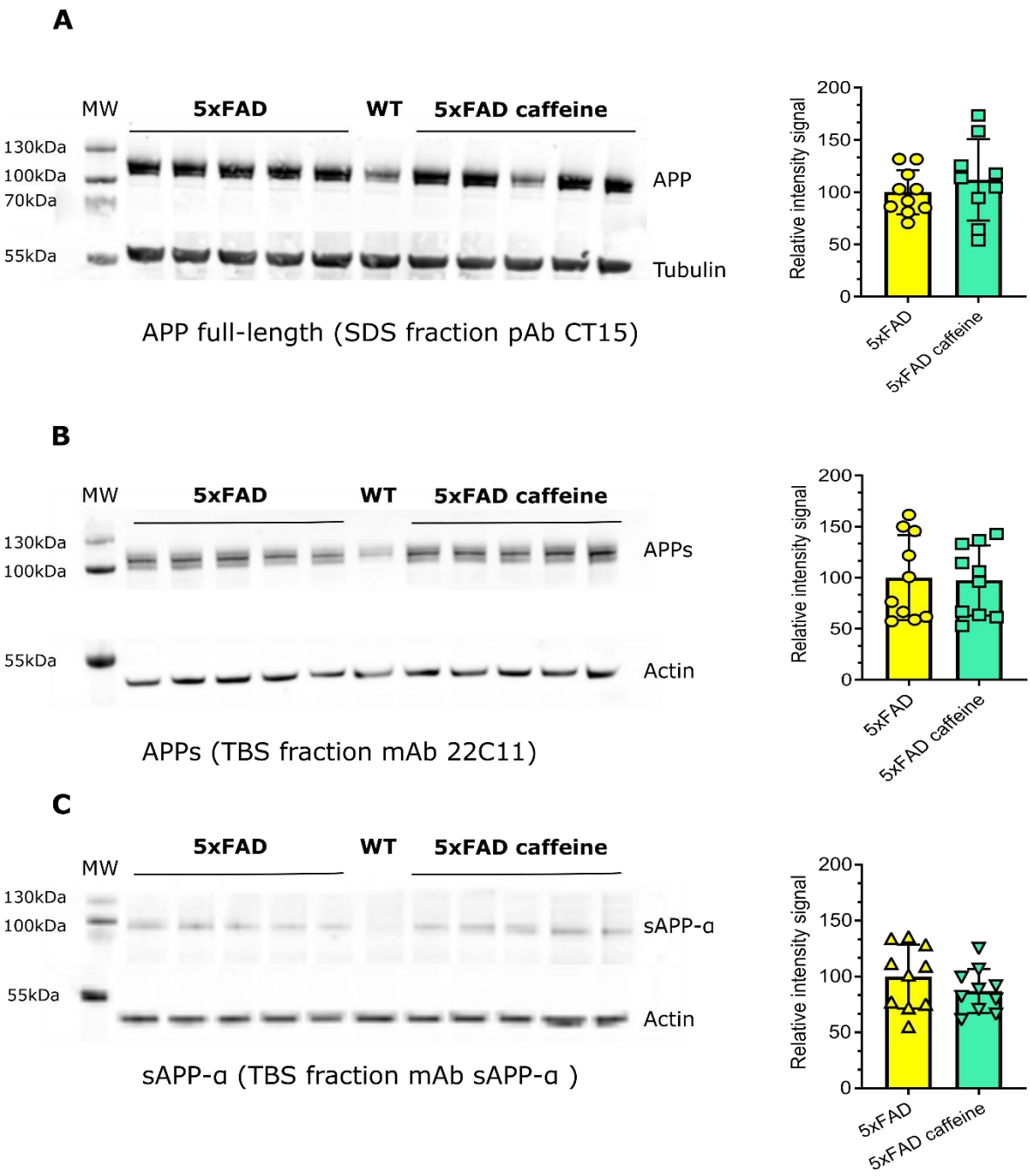

Figure 29: Analysis of APP processing in 5xFAD mice after chronic oral caffeine treatment.
(A) No changes were seen in the level of APP C-terminal fragments (CTFs) in SDS fractions between untreated and caffeine 5xFAD mice using antibody CT-15 directed against the APP C-terminus. (B) The same held trus for total secreted APP (APPs) in the TBS fraction using antibody 22C11 and (C) for the sAPP-α using an antibody detecting α-secretase cleaved APPs. WT mice lack the human APP-transgene and express only endogenous levels of murine APP. Unpaired t-test. 5xFAD n = 10 and 5xFAD caffeine n = 10. All data were given as mean ± SD.

3.3. PROJECT III Protective effects of chronic riluzole intake in Tg4-42^{hom} mice

Two-month-old Tg4-42^{hom} and WT mice were treated for 4 months with riluzole (25 mg/L) in drinking water (Figure 30A). At 6 months of age, mice were subjected to a battery of behavioural tests with ongoing treatment. At the end of the behavioural testing phase, animals were sacrificed for further analysis. To monitor the health status of the animals, body weight and liquid intake were recorded through the entire behavioural assessment phase. No differences in water consumption (Figure 30B) or body weight (Figure 30C) were observed among all groups, irrespective of the treatment.

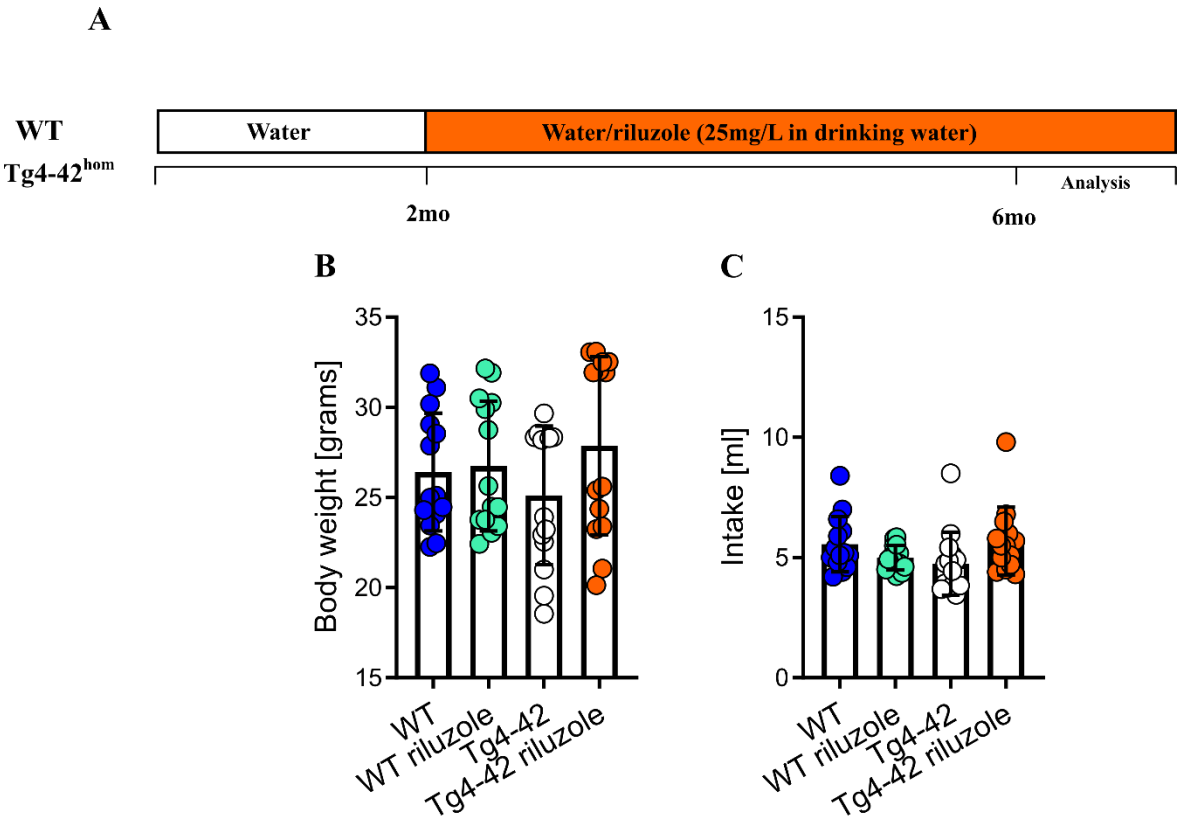

Figure 30: Experimental design and physiological status of animals after riluzole treatment.
(A) Experimental design. **(**B) Body weight and (C) daily water consumption assessment during the behavioural test analysis. (B) Riluzole treatment does not have an influence on the body weight of mice and (C) the daily water intake was similar between the groups. (B, C) One-way ANOVA followed by Bonferroni multiple comparison test. WT n = 14, WT riluzole n = 14, Tg4-42^{hom} n = 14, and Tg4-42^{hom} riluzole n = 14. Data are presented as mean ± SD.

3.3.1. Chronic riluzole treatment restores recognition and spatial memory deficits in Tg4-42^{hom} mice

As previously described, deficits in both recognition and spatial memory were evident in Tg4-42^{hom} mice (Antonios et al., 2015; Hüttenrauch et al., 2016a). To evaluate whether chronic oral riluzole treatment ameliorates these memory deficits, the NOR and the MWM were performed. The NOR test was used to analyse novelty preference and recognition memory. On day one, all the groups spent an equal amount of time exploring the two identical objects (Figure 31A). Twenty-four hours later, during the testing phase of the NOR, the Tg4-42^{hom} untreated group did not show any preference for the new presented object, while WT, the WT riluzole and the Tg4-42^{hom} riluzole-treated mice spent significantly more time exploring the novel object compared with the familiar one (Figure 31B, $p < 0.001$). This observation is confirmed by the calculation of the discrimination index (Figure 31C, $p < 0.001$).

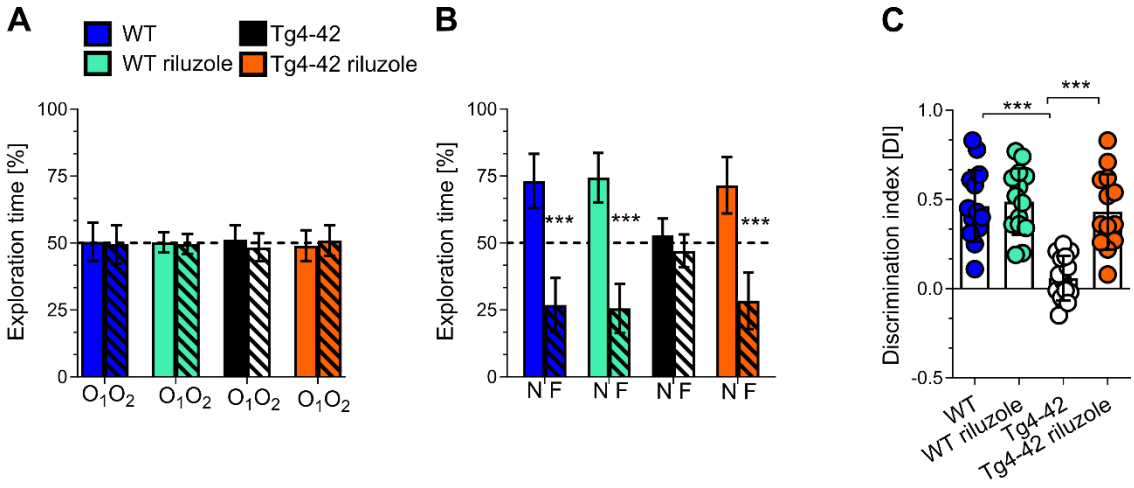

Figure 31: Riluzole treatment restores object recognition memory in the Tg4-42^{hom} mice.
(A) During the exploration phase on day one, all four groups spent an equal amount of time with each of the two identical objects (O_1, O_2). (B) During the test trial, WT as well as riluzole-treated WT and riluzole-treated Tg4-42^{hom} mice spent significantly more time with the novel object (N) while untreated Tg4-42^{hom} animals spent an equal amount of time with both objects. (A, B) Two-way ANOVA followed by Bonferroni multiple comparisons test. (C) One-way ANOVA followed by Bonferroni multiple comparisons test. *** $p < 0.001$. WT n = 14, WT riluzole n = 14, Tg4-42^{hom} n = 14, and Tg4-42^{hom} riluzole n = 14. Data are presented as mean ± SD.

The MWM test was used to evaluate the effects of riluzole on spatial reference memory deficits of the Tg4-42^{hom} mice. All mice showed progressively decreased escape latencies over the initial cued and acquisition training phases, training (Figure 32A and C respectively). Moreover, all the four groups displayed comparable swimming speeds during the entire training period (Figure 32B and D). Twenty-four hours after the last acquisition trial, the 60 s probe trial was performed to assess spatial reference memory. Six-month-old Tg4-42^{hom} mice displayed no significant preference for the target quadrant, while either WT controls, or riluzole-treated WT and Tg4-42 mice showed a significant target quadrant preference (Figure 32E, $p < 0.001$). Again, no difference in the swimming speeds was observed (Figure 32F).

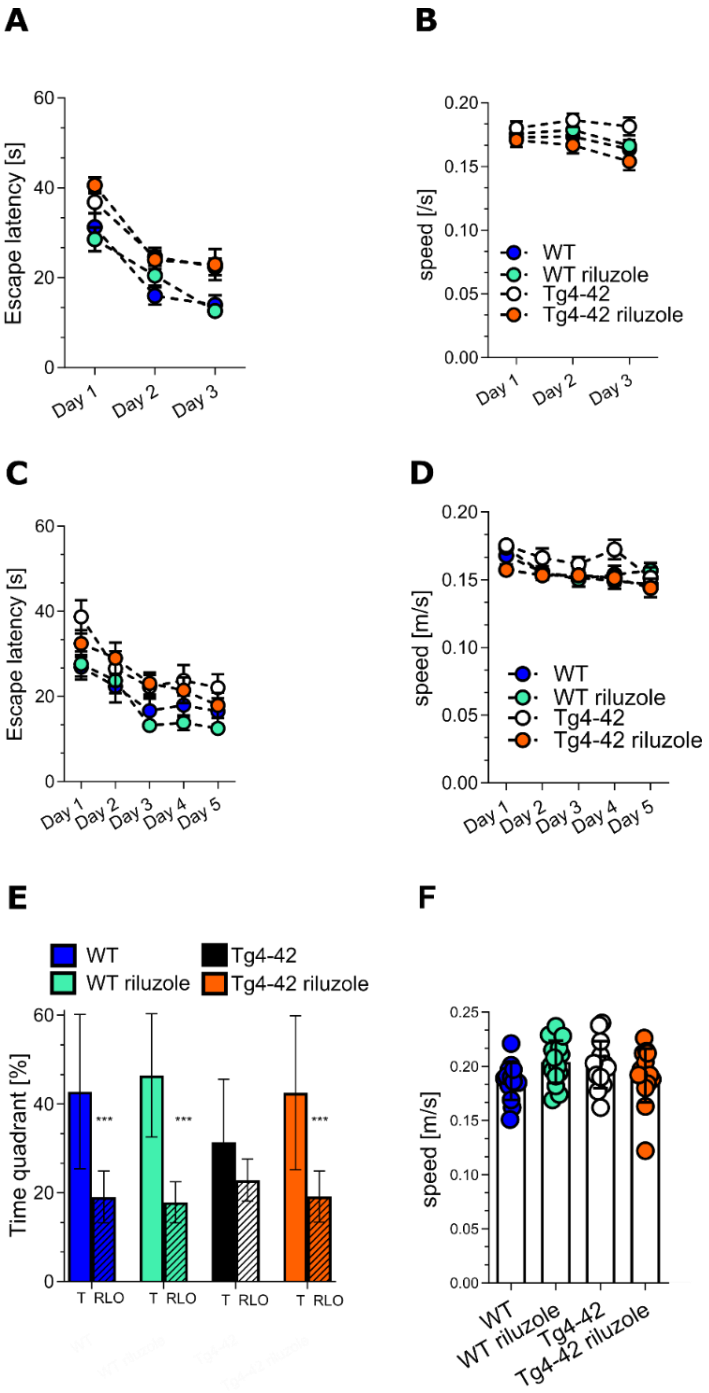

Figure 32: Impaired spatial reference memory in Tg4-42^{hom} mice is restored upon riluzole treatment.
(A and C) WT and Tg4-42^{hom}, as well as riluzole-treated WT and Tg4-42^{hom} mice showed decreased escape latencies over the 3 and 5 days of cued and acquisition training. (B and D) No differences in swimming speed were observed between all the four groups in both cued and acquisition training. (**E**) Tg4-42^{hom} mice showed no preference for the target quadrant (T) during the probe trial. Both vehicle- and riluzole-treated WT, as well as Tg4-42^{hom} riluzole-treated mice displayed an intact spatial reference memory, spending significantly more time in the target quadrant (T) compared to the average of the other quadrants (RLO). (F) During the probe trial, no difference in swimming speeds was observed. (A-E) Two-way ANOVA followed by Bonferroni multiple comparison test. (F) One-way ANOVA followed by Bonferroni multiple comparisons test. ***$p < 0.001$. WT n = 14, WT riluzole n = 14, Tg4-42^{hom} n = 14, and Tg4-42^{hom} riluzole n = 14. All data were given as mean ± SD.

3.3.2. Riluzole ameliorates hippocampal neuron loss in the CA1 area and restores impaired neurogenesis in the dentate gyrus of Tg4-42^{hom} mice

To analyse if a chronic oral riluzole intake has an impact on CA1 neuron numbers in Tg4-42^{hom} mice, a quantification of the number of haematoxylin-stained neuronal nuclei in a defined area of the hippocampal CA1 region in 6-month-old untreated and riluzole-treated WT and Tg4-42^{hom} mice was carried out. In agreement with a previous study (Antonios et al., 2015), 6-month- old Tg4-42^{hom} present with ~50% neuron loss compared with aged-matched WT animals (Figure 33, $p < 0.001$). In comparison to the untreated WT control group, riluzole-treated Tg4-42^{hom} mice displayed a neuron loss of ~38% that was significantly different from the untreated Tg4-42^{hom} group (Figure 33, $p < 0.05$). Moreover, a stereological analysis of dentate gyrus DCX-positive cells revealed a significant and strongly reduced number of DCX-positive neurons in the DG of the hippocampus in the 6-month-old Tg4-42^{hom} mice compared to the WT mice, as previously shown in Figs 8 and 14 (Figure 34, $p < 0.001$). Interestingly, long-term oral riluzole consumption normalized the numbers of DCX-positive cells in Tg4-42^{hom} riluzole-treated mice to WT levels (Figure 34, $p < 0.001$).

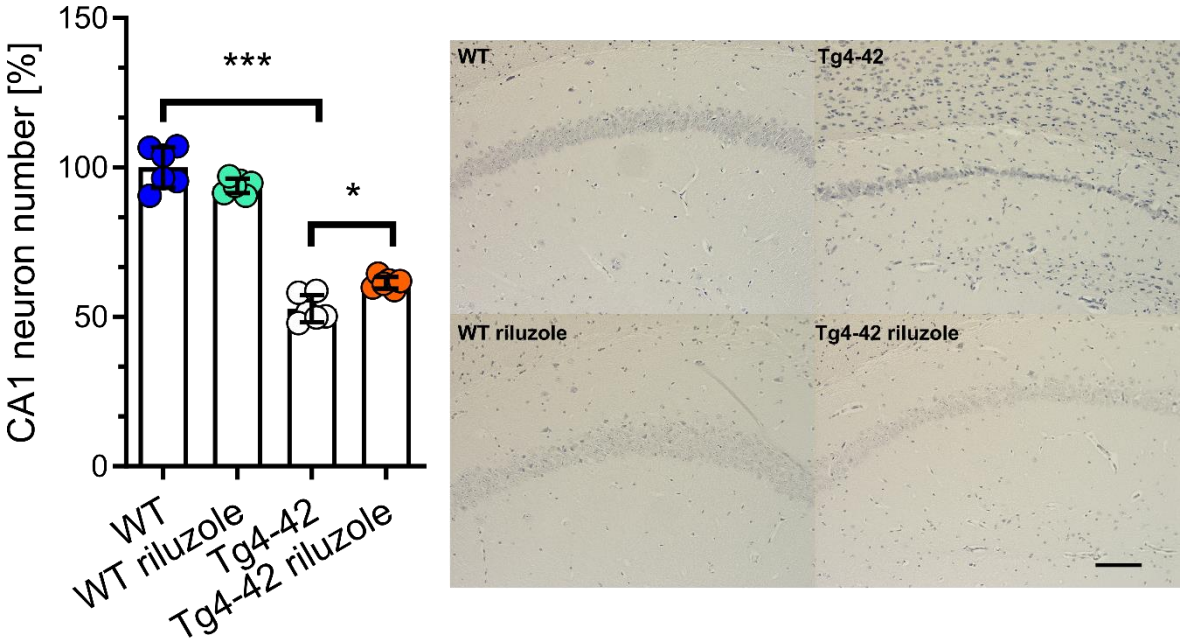

Figure 33: Riluzole treatment decreases hippocampal neuronal loss in the CA1 area in Tg4-42^{hom} mice.
(A) Analysis of hematoxylin-stained sections revealed significantly reduced CA1 neuron numbers in Tg4-42^{hom} mice compared to WT (-~50%) and an ameliorated neuron loss after riluzole treatment (-~38%). Two-way ANOVA followed by Bonferroni multiple comparison tests. *p < 0.05, ,****p* < 0.001.WT n = 6, WT riluzole n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} riluzole n = 6. All data were given as mean ± SD. Scale bar = 50 µm.

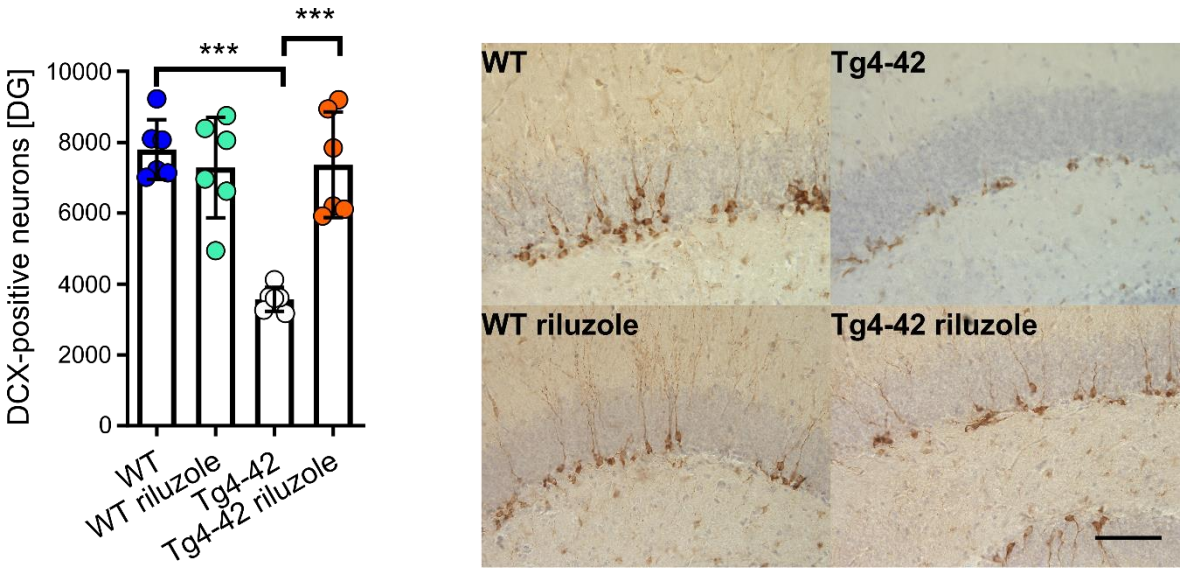

Figure 34: Riluzole treatment increases neurogenesis in the Tg4-42^{hom} mice.
Analysis of doublecortin (DCX)-stained sections revealed a significantly reduced number of DCX-positive cells in Tg4-42^{hom} mice compared to WT control mice. Chronic oral riluzole intake restores the number of new-born neurons in Tg-4-42^{hom} riluzole mice to WT levels. Two-way ANOVA followed by Bonferroni's multiple comparison test. ****p* < 0.001. WT n = 6, WT riluzole n = 6, Tg4-42^{hom} n = 6, and Tg4-42^{hom} riluzole n = 6. All data are given as mean ±SD. Scale bar = 50 µm.

3.4. PROJECT IV: Physical activity and cognitive stimulation ameliorate learning and motor deficits in Tg4-42^{hom} mice[2]

Tg4-42^{hom} mice were randomly assigned to either standard housing (SH) or environmental enriched (EE) housing conditions at an age of two months for a duration of four months. At the end of either housing paradigm, mice were subjected to behavioural testing and sacrificed for further analysis (Figure 35).

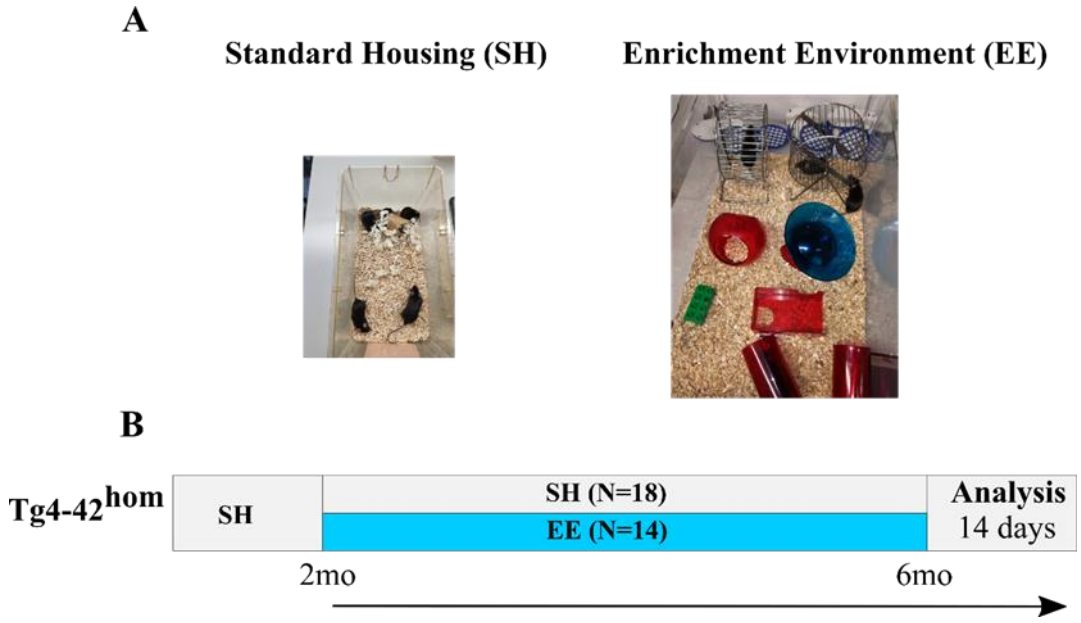

Figure 35: Housing conditions and experimental design.
(A) Exemplary images of standard housing (SH) and environmental enrichment (EE) cages used in this project. Mice were housed in groups of 3-4. EE cages were equipped with colourful tunnels, climbing toys and three running wheels (2 metal, 1 plastic). Once a week the environment was cleaned, modified, and rearranged completely. (B) At 2 months of age, Tg4-42^{hom} were randomly assigned to either SH or EE housing conditions for 4 months. At 6 months of age, mice were behaviourally analysed, sacrificed and tissue was collected.

3.4.1. Environmental enrichment improves motor skills in Tg4-42^{hom} mice

To evaluate the effect of EE housing on the motor skills, Tg4-42^{hom} mice underwent to two different motor tasks. With the rotarod task, general motor skills were analysed. Over the 8 trial sessions of the two consecutive days of testing, both groups improved their ability to stay on the rod. However, Tg4-42^{hom} EE performed significantly better on the rotarod

[2] The results presented in this chapter are published in **Stazi M**, Wirths O. Physical activity and cognitive stimulation ameliorate learning and motor deficits in a transgenic mouse model of Alzheimer's disease. Behav Brain Res. 2021 Jan 15;397:112951. doi: 10.1016/j.bbr.2020.112951. Epub 2020 Oct 4. PMID: 33027669.

compared to SH mice as demonstrated by overall higher latencies to fall (Figure 36A, $p < 0.001$). In addition, when tested for balance in the balance beam test, Tg4-42^{hom} EE mice performed significantly better than SH mice (Figure 36B, $p < 0.001$).

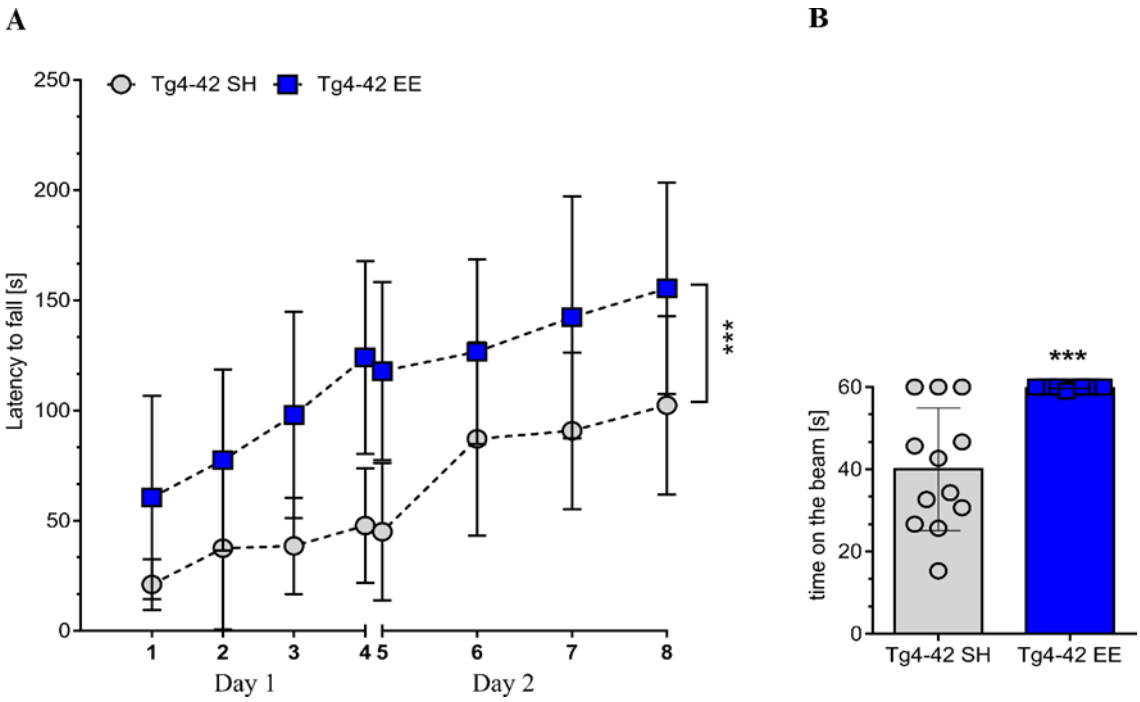

Figure 36: Effects of EE on sensory-motor performance of Tg4-42^{hom} mice.
(A) In the rotarod test, Tg4-42^{hom} EE showed significantly higher latencies to fall compared to SH mice. (B) In the balance beam test, Tg4-42^{hom} EE mice stayed significantly longer on the beam when compared to SH mice. (A) Two-way ANOVA followed by Bonferroni multiple comparisons test. (B) Unpaired t-test ***$p < 0.001$. Tg4-42^{hom} SH n = 12 and Tg4-42^{hom} EE n = 14. All data were given as mean ± SD. Figure adapted from (Stazi and Wirths, 2021b).

3.4.2. EE housing improves recognition and working memory performance in Tg4-42^{hom} mice

Anxiety levels and general locomotor activity in Tg4-42^{hom} SH and EE mice was assessed using the OF test (Figure 37A). Tg4-42^{hom} EE mice passed-through significantly more seconds in the centre of the OF arena compared with SH control animals (Figure 37B, $p < 0.001$), indicating an increased exploratory and reduced anxiety behaviour, although no difference in the total distance travelled was found (Figure 37C).

Recognition memory was evaluated using the NOR task (Figure 37A). On the exploration day (day1), Tg4-42^{hom} SH and EE mice explored the two indistinguishable items for an equal percentage of time (Figure 37D), demonstrating no basal difference in levels of curiosity and

general interest in exploring novel objects. When tested for recognition memory twenty-four hours later, as expected, Tg4-42^{hom} SH mice did not show any object preference (Figure 37E), while EE mice spent significantly more time with the novel object (N) compared to the familiar one (Figure 37E, $p < 0.001$). The analysis of the DI validated this finding with EE mice showing significantly highest DI scores compared to SH littermates (Figure 37F).

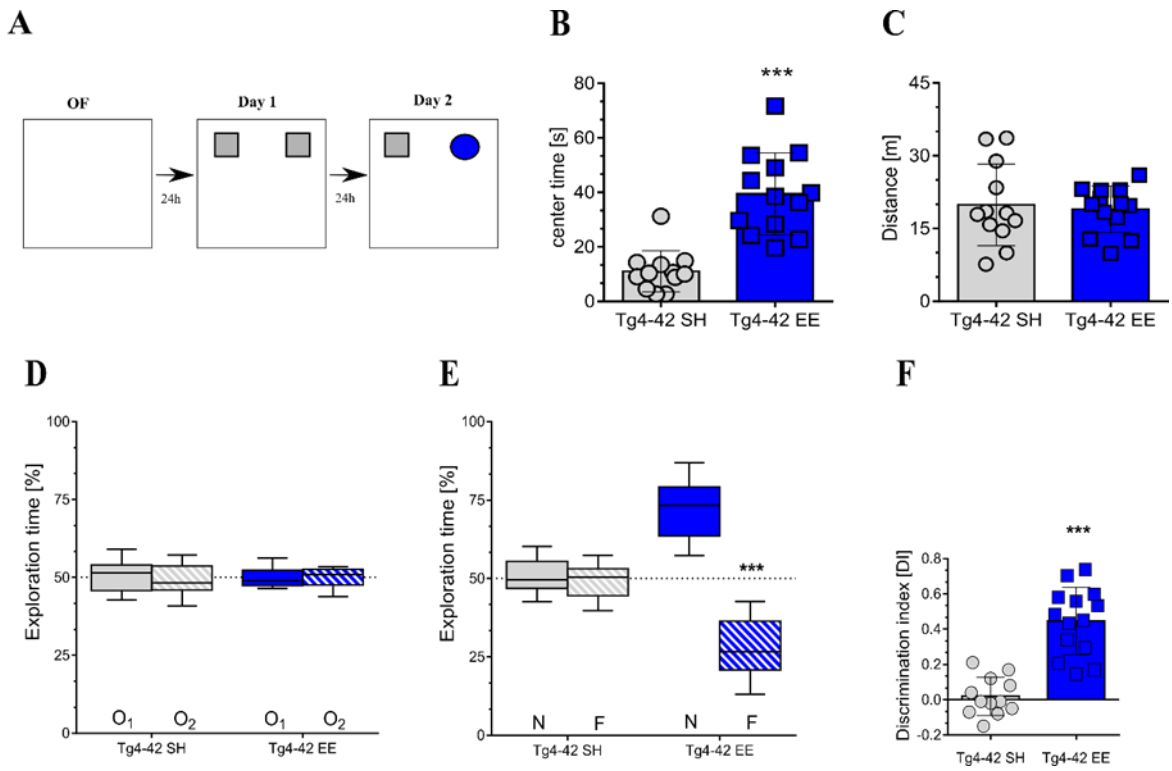

Figure 37: EE housing restores recognition memory deficit in Tg4-42^{hom} mice.
The NOR test was used to evaluate recognition memory. (A) Schematic drawing of the NOR task. (B) During the open field task Tg4-42^{hom} EE mice spent more time in the centre of the OF compared with SH mice; (C) while no differences in distance travelled was detected between the two groups. (D) On day 1 of the NOR, SH and EE mice spent an equal amount of time with each of the two indistinguishable objects. (E) After 24 h, during the test trial, EE mice showed a clear preference for the novel object (N), whereas no object preference was observed for the SH group. (F) The DI calculation validates this observation, with the Tg4-42^{hom} EE animals having a significantly higher DI compared to the SH mice. (B, C, F) Unpaired t-test. (D, E) Two-way ANOVA followed by Bonferroni multiple comparisons test. ***$p < 0.001$. Tg4-42^{hom} SH n=12 and Tg4-42^{hom} EE n=14. All data were given as mean ± SD. (O_1 = Object 1 and O_2 = Object 2; N = Novel object and F = Familiar object). Figure adapted from (Stazi and Wirths, 2021b).

The Cross-Maze test was used to assess if the housing condition had an effect on hippocampus-related spatial working memory. In general, Tg4-42^{hom} mice at six-months of age did not display deficits in this task as they performed better than chance level (indicated by the dotted line). Nevertheless, mice housed in EE showed increased alternation rates compared with SH mice, meaning a significantly improved performance (Figure 38A, $p <$

0.05). No differences in total arm entries were observed between the two groups, meaning that the better performance of Tg4-42^{hom} EE mice is not simply due to altered explorative behaviour (Figure 38B).

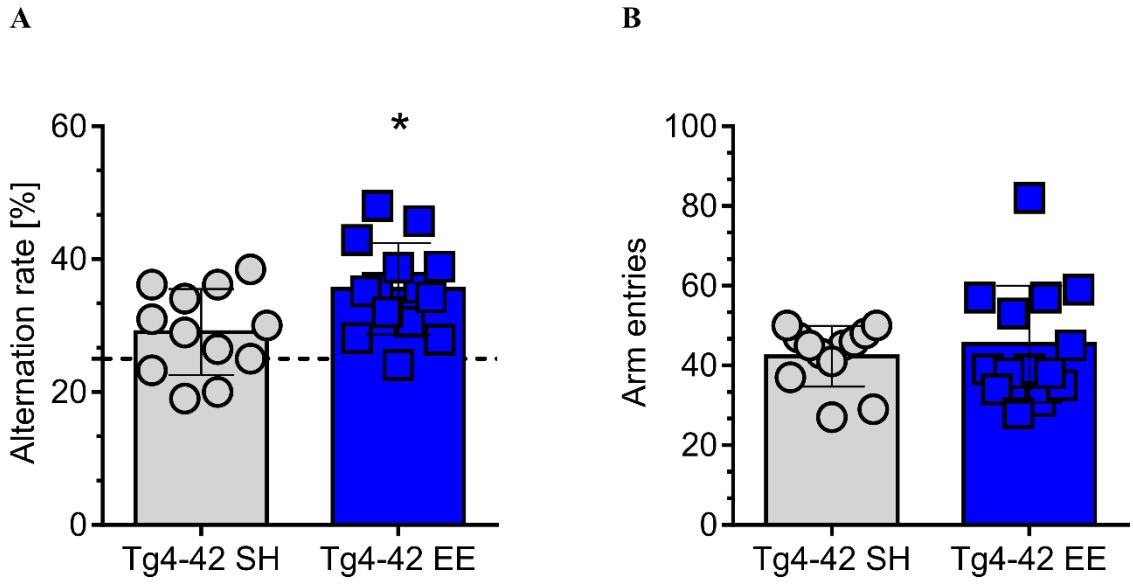

Figure 38: Environmental enrichment improves working memory in Tg4-42^{hom} mice.
(A) In the cross-maze task, Tg4-42^{hom} EE mice presented significantly higher alternation rates respect the SH group. The dotted line represents chance level. (B) No difference in the total arm entries was find between the two groups. Unpaired t-test. *$p < 0.05$. Tg4-42^{hom} SH n=12 and Tg4-42^{hom} EE n=14. All data were given as mean ± SD. Figure adapted from (Stazi and Wirths, 2021b).

3.4.3. Enriched environment housing ameliorates spatial memory deficits in the Morris water maze

Spatial reference memory of was assessed using the Morris water maze (MWM). Here, we confirmed our previous findings (Hüttenrauch et al., 2016a) in a completely independent and different cohort (using both gender) and in addition with a variety of novel analysis parameters. Both groups of mice showed strongly decreased escape latencies over the initial training phase (Figure 39A and C representing the 3-days of cue training and 5-days of acquisition training respectively). However, Tg4-42^{hom} EE animals presented significantly shorter escape latencies over the entire training period compared to their SH control group (Figure 39A $p < 0.05$ in the cued and in the acquisition Figure 39C $p < 0.01$). This indicates improved spatial learning upon prolonged enrichment environment housing already during

the training phase. No differences in swimming speed were noticed during the whole training period between both groups (Figure 39B cue training and D acquisition training). In the probe trial, while SH mice did not present a target quadrant preference, mice housed under enriched conditions showed an intact spatial reference memory, spending significantly more time in the target quadrant (T) in comparison to the average time of the other three quadrant (RLO) (Figure 39E, $p < 0.001$). Representative occupancy plots during the 1-min probe trial evidenced that EE mice focused their search on the platform location while sedentary mice explored the quadrants in a more random way (Figure 39E). Again, no difference in swimming speed was noted in Tg4- 42^{hom} SH/EE mice in the final probe trial (Figure 39F).

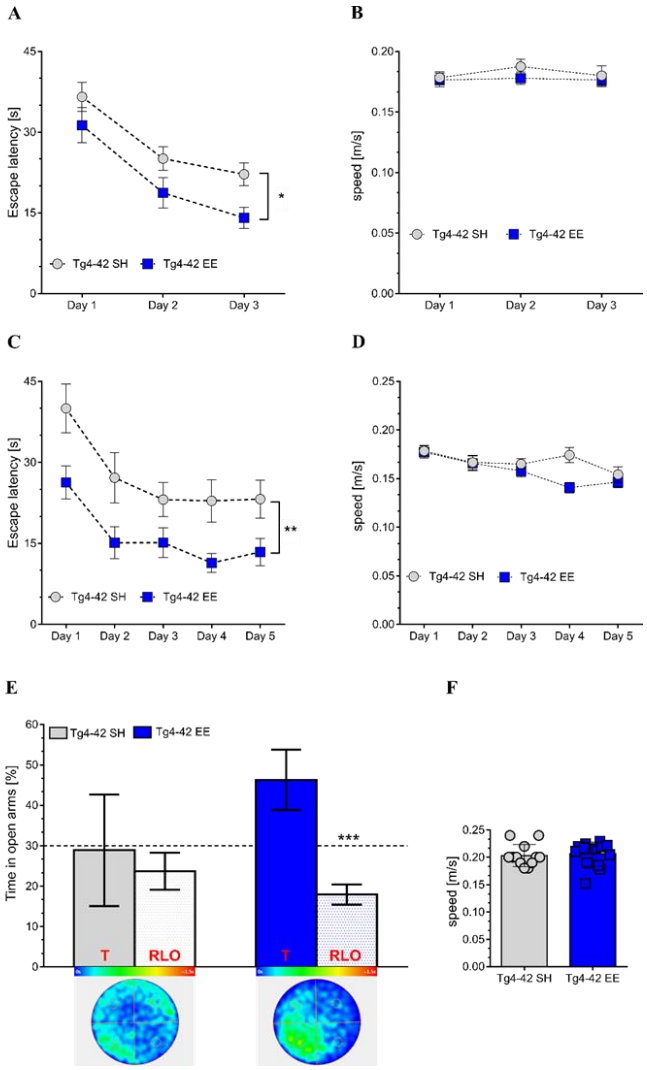

Figure 39: EE housing restores impaired spatial reference memory in Tg4-42^{hom} mice.
(A and C) Tg4-42^{hom} SH and EE mice showed decreased escape latencies over the cued and acquisition training. However, Tg4-42^{hom} EE mice displayed a significantly better performance than SH mice as shown by overall lower escape latencies during the entire duration of the training period. (B, D) No differences in swimming speed were observed between Tg4-42^{hom} SH/EE mice in both cued and acquisition training. (E) Tg4-42^{hom} SH mice showed no preference for any of the quadrants during the probe trial, while the EE mice displayed an intact spatial reference memory as they spent significantly more time in the target quadrant (T) compared to all the other quadrants (LRO). The occupancy plots indicate exemplarily the averaged swimming traces during the probe trial. (F) During the probe trial, again no difference in swimming speed was find. (A-E): Two-way ANOVA followed by Bonferroni multiple comparisons test. (F): Unpaired t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Tg4-42^{hom} SH n=12 and Tg4-42^{hom} EE n=14. All data were given as mean ± SD (T= Target, L = Left, R = Right, O = Opposite). Figure adapted from (Stazi and Wirths, 2021b).

A deep evaluation of swimming behaviour during the probe trial revealed that EE housing improved spatial reference memory in several different parameters. EE-housed mice showed significantly more entries (Figure 40A, $p < 0.05$) and time spent in the former platform position (Figure 40B, $p < 0.01$) as well as target quadrant entries (Figure 40D, $p < 0.001$) and more time swam in the target quadrant respect the SH-mice (Figure 40E, $p < 0.001$). Furthermore, EE-housed animals displayed decreased latencies to the first entry of platform

and target quadrant compared to the SH control group (Figure 40C $p < 0.01$ and F $p < 0.001$ respectfully).

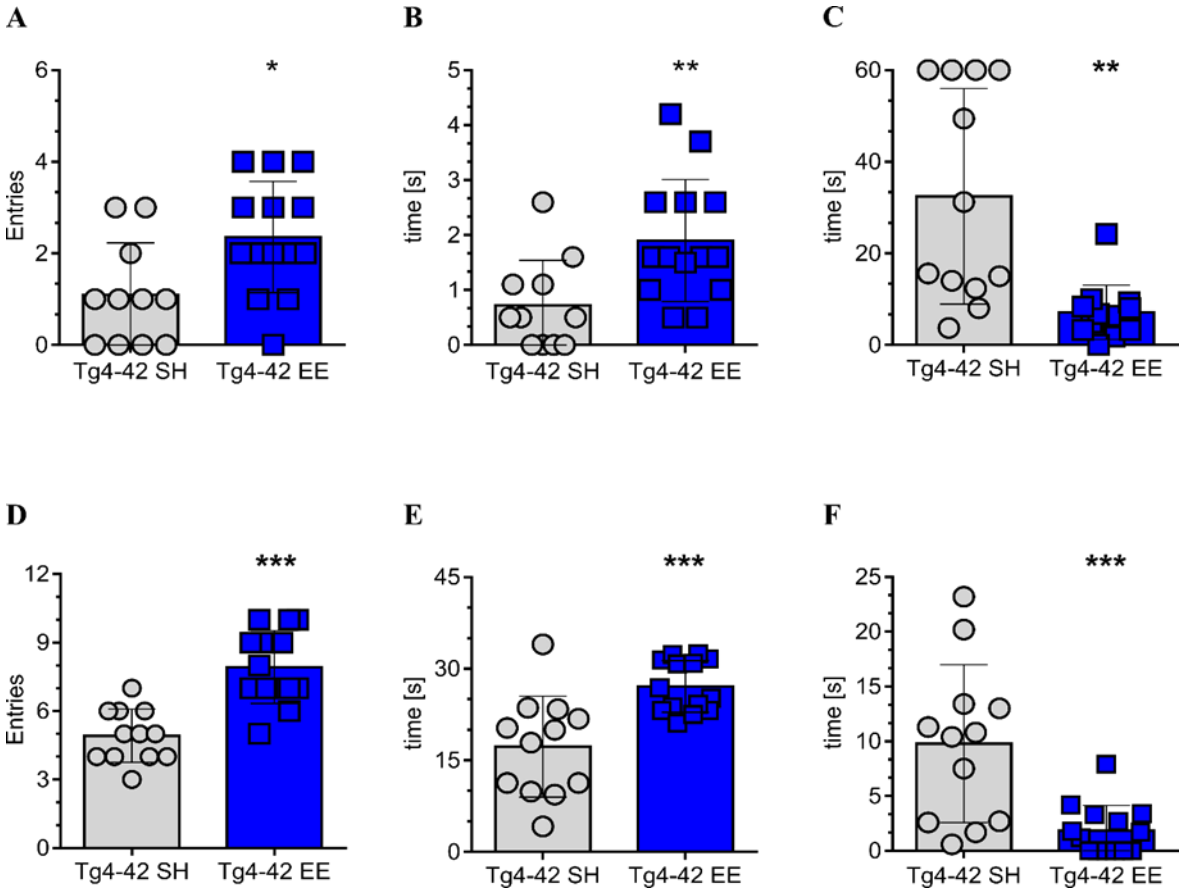

Figure 40: EE housing improves spatial reference memory in the Tg4-42^{hom} mice.
The number of platform and target quadrant entries (A, C), the time spent in the platform and target zone (B, D) and the latency to first enter the location of the target quadrant (E) or the original platform position (F) during the probe test were evaluated. Mice housed under EE showed a better performance in all parameters analysed. (A-F) Unpaired t- test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Tg4-42^{hom} SH n=12 and Tg4-42^{hom} EE n=14. All data were given as mean ± SD. Figure adapted from (Stazi and Wirths, 2021b).

3.4.4. Enriched environment housing rescues the floating phenotype in Tg4-42^{hom} mice

During the MWM test, we observed that some of the mice spent time floating in the water rather than swimming in the pool to find the platform We described “floating behaviour” as times of immobility in the water of at least 0.5 sec. (McBrayer et al., 2015). A more accurate analysis of this phenomenon disclosed that the Tg4-42^{hom} mice displaying floating behaviour presented a significantly reduced swimming speed respect to non-floaters animals (Figure 41A, $p < 0.001$). This finding was validated by analysing the immobility time and immobility

episodes, which were significantly different between non-floating and floating Tg4-42^{hom} animals (Figure 41B and C, $p < 0.05$). Interestingly, none of the mice housed under EE conditions presented this floating phenotype, meaning a significant effect of the housing condition (Figure 41D and E, One sample Wilcoxon test, both $p < 0.05$).

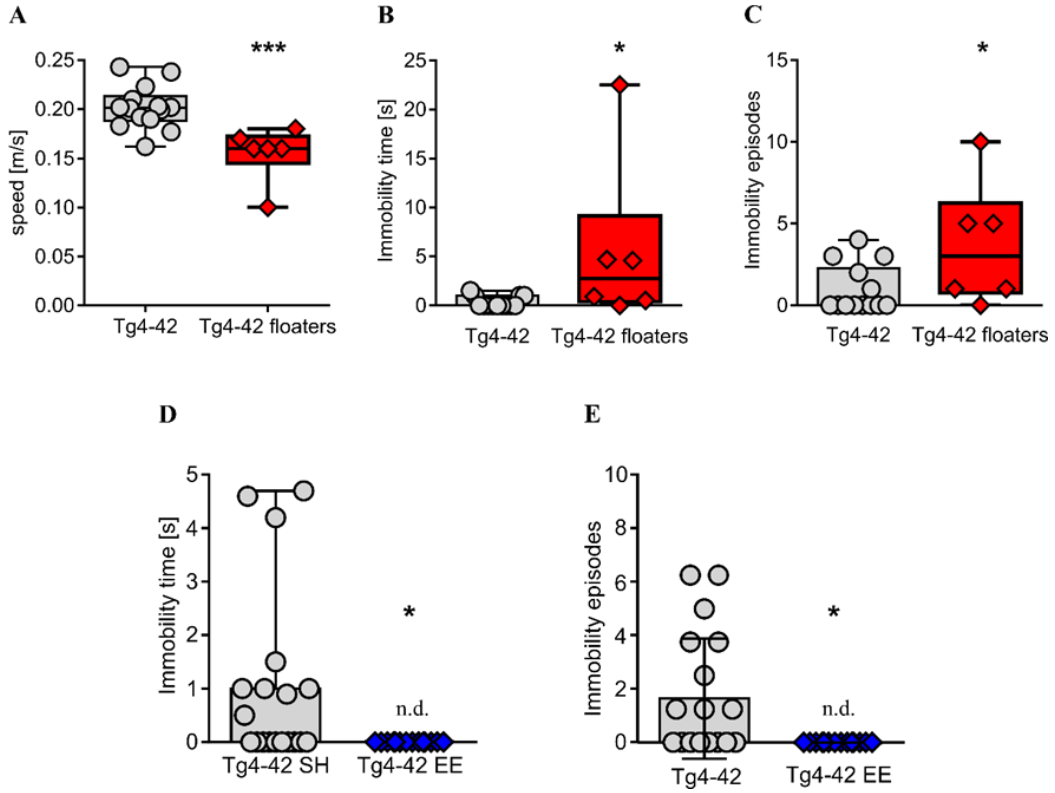

Figure 41: Floating behaviour phenotype in the probe trial of MWM of Tg4-42^{hom} mice is rescued after EE housing. (A) The floating behaviour of the Tg4-42^{hom} has an impact on the average swimming speed during the probe trial of the MWM. Evaluation of the immobility time (B) and immobility episodes (C) confirmed the phenotype. After the EE housing, the floating phenotype of the Tg4-42^{hom} was completely rescue as the EE group did not show any (D) immobility time or (E) immobility episodes. (A, B, C) Unpaired t-test. (D, E) One sample Wilcoxon test. *$p < 0.05$; ***$p < 0.001$. Tg4-42^{hom} SH n=18 (one male and five females were floaters) and Tg4-42^{hom} EE n=14. All data were given as mean ± SD. n.d. no date. Figure adapted from (Stazi and Wirths, 2021b).

3.4.5. The effect of enriched environment housing on Aβ levels and hippocampal neuron loss in Tg4-42^{hom} mice

One of the key characteristic of the Tg4-42 mouse model is the overexpression of the Aβ$_{4-42}$ that, starting from 2 month of age, lead to a strong intraneuronal immunoreactivity mainly in the CA1 region of the hippocampus (Bouter et al., 2013). This Aβ accumulation well correlate with the age-dependent neuronal loss (Antonios et al., 2015), however due to this severe neuronal death the immunoreactivity of the Aβ decline throughout the age (Bouter et

al., 2013). To evaluate whatever the housing condition influence $A\beta_{4-42}$ levels, immunohistochemical analysis, using an $A\beta_{42}$ specific antibody D3E10, were done in Tg4-42^{hom} SH and EE. The quantification of intraneuronal Aβ reveal that housing in a stimulating environment did not alter the Aβ levels, since no significant difference were detected between SH o EE mice (Figure 42A). Despite that the haematoxylin staining analysis of the CA1 area showed an ~20% less decline of CA1 neuron numbers in Tg4-42^{hom} EE mice respect to the SH littermates (Figure 42B, $p < 0.01$).

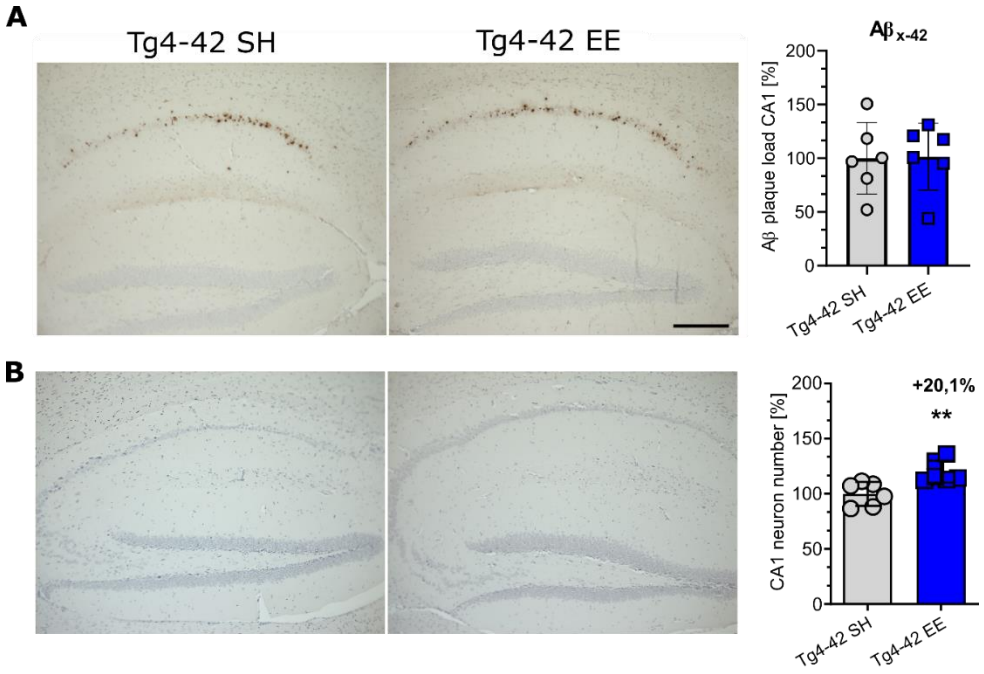

Figure 42:The effect of enrichment environment housing on Aβ levels and CA1 neuronal loss.
(A) No changes in Aβ deposition were detected using an $A\beta_{42}$-specific antibody in an immunohistochemical analysis in the hippocampal CA1 layer upon housing under enriched environment conditions in Tg4-42^{hom} mice. (B) However, quantification of CA1 neuron numbers in 6-months old Tg4-42^{hom} showed a diminished neuron loss (~20 %) in Tg4-42^{hom} EE mice. Unpaired t-test, $p < 0.01$. Tg4-42^{hom} SH n = 6 and Tg4-42^{hom} EE n = 6 Scale bar: 200 µm.

4. DISCUSSION

Alzheimer's disease represents the most common cause of dementia worldwide. The development of research models, especially rodents, is becoming to be a crucial research priority as they can be a very useful tool to understand the pathomechanisms of neurodegeneration and investigate preventive strategies and/or treatments. In the present dissertation, two mouse models of AD have been studied: (i) Tg4-42, representing a transgenic model for the sporadic form of the disease, and (ii) 5xFAD, a model for familial AD. Currently, there is no effective therapy for AD, and in this work three different drug treatments, as well as an environmental intervention as potential preventive therapy have been investigated.

The first three projects aimed to study the effects of preventive and therapeutic chronic oral intake of memantine, caffeine and riluzole, with regard to behavioural impairment, altered neurogenesis and CA1 neuron loss in 6-month-old Tg4-42^{hom} mice. Moreover, caffeine treatment was also applied to address its potential effects on memory deficits, Aβ plaques deposition, neuroinflammation and APP processing in 6-month-old 5xFAD mice.

Lastly, the effects of enriched environment living conditions were investigated with a focus on sensory-motor deficits and general cognition in 6-month-old Tg4-42^{hom} mice.

It is important to note that all the treatment approaches started prior to disease onset at 2 months of age for both mouse models and continued for four months. At this time point Tg4-42^{hom} mice displayed severe behavioural deficits and robust neuron loss, and 5xFAD showed widespread Aβ plaque deposition in many brain regions.

In the following sections of this dissertation, the different results of this work will be discussed.

4.1. PROJECT I: Chronic memantine treatment delays hippocampal neurodegeneration and rescues memory deficits in the Tg4-42^{hom} mice

Currently, only two classes of drugs are approved to treat AD, which are acetylcholinesterase inhibitors and the NMDA receptor antagonist memantine (Graham et al., 2017). While for example donepezil or rivastigmine (acetylcholinesterase inhibitors), are approved for all disease stages, memantine is only authorized for moderate to severe AD. Treatments with either cholinesterase inhibitors or memantine seem to have statistically significant improvement in measures of cognition and global assessment of dementia (McShane et al., 2019; Raina et al., 2008).

One of the key hallmarks in the pathomechanism of AD is the severe decrease in the numbers of neurons in various regions of the brain (Mukhin et al., 2017). In particular, a drastic neuronal loss in the hippocampal CA1 region and the entorhinal cortex, two brain regions associated with the severity of memory impairments, is reported (Giannakopoulos et al., 2003). As a difficulty to study the extent of neurodegeneration, only a limited number of AD mouse models showed a reliable hippocampal neuron loss (Morrissette et al., 2009; Wirths and Bayer, 2010, 2012; Wirths and Zampar, 2020). Tg4-42^{hom} mice show a significantly reduced number of CA1 pyramidal neurons at six months of age, in a range of approximately around 50% compared to age-matched WT littermates (Antonios et al., 2015). Moreover, stereological investigations confirmed that the progressive neuron loss in the hippocampus of Tg4-42 mice begins at four months of age and correlates strongly with intraneuronal A$\beta_{4\text{-}42}$ expression (Antonios et al., 2015; Bouter et al., 2013).

Memantine has been reported to exert neuroprotective effects against β-amyloid-induced neurodegeneration, as described by Miguel-Hidalgo et al. (Miguel-Hidalgo et al., 2002), showing that vehicle-treated rats with intra-hippocampal injections of A$\beta_{1\text{-}40}$ presented more severe neuronal damage in the CA1 region compared with rats treated with memantine (Miguel-Hidalgo et al., 2002).

In addition, memantine-treated rats injected with $A\beta_{1-40}$ or $A\beta_{1-42}$ displayed a significant preservation of cholinergic neurons in the basal forebrain compared to untreated control groups (Castaneda et al., 2015; Nyakas et al., 2011). In good agreement with these findings, analysis of haematoxylin-stained sections revealed a significant amelioration of CA1 pyramidal neuronal loss in memantine-treated Tg4-42^{hom} in the current thesis (Figure 16) (Stazi and Wirths, 2021a). In particular, an analysis differentiating between the distal and the proximal parts of the hippocampal CA1 area disclosed that the severe neuronal loss observed at six months was reduced in the distal CA1portion of the drug-treatment animals, as memantine-treated Tg4-42^{hom} mice presented ~17% more neurons compared to the untreated group (Figure 16) (Stazi and Wirths, 2021a).

Impaired adult neurogenesis is a common characteristic of transgenic AD mice and is present in a variety of models (Wirths, 2017), such as 3xTg-AD (Rodríguez et al., 2008), APP/PS1KI (Cotel et al., 2012), Tg2576 (Krezymon et al., 2013), and 5xFAD (Moon et al., 2014). There is a vast body of literature describing that memantine treatment facilitates adult neurogenesis in rodent brain (Akers et al., 2014; Ishikawa et al., 2019; Jin et al., 2006; Maekawa et al., 2009). This beneficial effect was noted even after a single intra-peritoneal injection of 50 mg/kg of memantine with new-born cells differentiating into mature granule cells (Maekawa et al., 2009).

Six-month-old Tg4-42^{hom} mice display a strongly reduced neurogenesis in the DG region of the hippocampus (Gerberding et al., 2019) when compared to WT littermates. Next to the decreased CA1 neuron loss described previously, a significant amelioration of neurogenesis was observed in 6-month-old memantine-treated Tg4-42^{hom} mice (Figure 17) (Stazi and Wirths, 2021a).

Alongside the cognitive decline, AD patients frequently also show non-cognitive symptoms like sensory-motor deficits (Buchman and Bennett, 2011), even if they are relatively less investigated (Albers et al., 2015). Alterations in motor performance seem to appear already quite early in the disease process (Buchman and Bennett, 2011), and these deficits have been also reported in a variety of transgenic mouse models, such as 5xFAD (Jawhar et al., 2012; Wirths and Bayer, 2008; Wirths et al., 2010) including Tg4-42 (Wirths and Bayer, 2010).

While there is meaningful data on the effects of memantine on cognition in different transgenic AD mouse models, only a limited number of studies with regard to motor performance is available. In good agreement with our results (Figure13A) (Stazi and Wirths, 2021a), oral memantine treatment did not improve rotarod performance in the Ts65Dn mouse model of Down syndrome (Rueda et al., 2010) or the G93A SOD1 mutation model of ALS (Joo et al., 2007). On the other hand, low-dose memantine treatment of a Huntington's disease mouse model results in improved performance in the rotarod task (Okamoto et al., 2009) and in a pilot study trial, 20mg/kg memantine treatment improved motor scores in Huntington's disease patients (Ondo et al., 2007). Even though we did not observe an amelioration in the rotarod task, deficits in the balance beam are completely rescued (Figure 13B) (Stazi and Wirths, 2021a). One possible explanation of this controversial results could be that while both tasks assess general motor functions, the balance beam task seems to provide a more sensitive measure of certain subtle motor skills, such as balance coordination (Stanley et al., 2005).

Several studies analysed the effects of memantine treatment on neuropathological changes as well as behavioural deficits in transgenic AD mouse models (Devi and Ohno, 2016; Dong et al., 2008; Filali et al., 2011; Liu et al., 2014; Martinez-Coria et al., 2010; Minkeviciene et al., 2004; Neumeister and Riepe, 2012; Scholtzowa et al., 2008; Zhou et al.,

2019); however, differences in drug administration and dosage, treatment duration, and the mouse models selected for the studies complicated to achieve common conclusions. Administration of memantine led to a significant decrease of amyloid plaque pathology in Tg2576 mice (six months of treatment in doses of 5 up to 20 mg/kg) (Dong et al., 2008) and 3-month-old 5xFAD (daily dose of 10 mg/kg for one month) (Jürgenson et al., 2019). In contrast, others found no effect on $A\beta_{42}$ levels in 6–7-month-old 5xFAD mice (Devi and Ohno, 2016).

Most of the used mouse models overexpressed APP and/or PSEN1 harbouring familial AD-associated mutations to achieve deposition of Aβ peptides forming extracellular plaques (Morrissette et al., 2009). While these mice usually exhibited AD-related pathological alterations, memory decline has been demonstrated in APP transgenic mice even without Aβ accumulation, indicating that other, non-AD-related confounding factors might play an important role in disease progression (Balducci and Forloni, 2011; Sasaguri et al., 2017). As mentioned above, in this thesis we used the Tg4-42 mouse model of AD, overexpressing only human $A\beta_{4\text{-}42}$ without overt plaque formation. Using this model, we can study the effects of the specific peptide without any interference from other human APP processing products. As mentioned before, Tg4-42 mice over-express $A\beta_{4\text{-}42}$ and harbour significant CA1 pyramidal neuron loss correlating with memory deficits. Previous data reported that 6-month-old Tg4-42^{hom} mice displayed a recognition memory deficit, assessed by the novel object recognition task (Hüttenrauch et al., 2016a), a commonly used behavioural test to evaluate recognition memory in rodents (Antunes and Biala, 2012). In comparison with age-matched WT, Tg4-42^{hom} mice showed a recognition memory deficit that was completely rescued upon long-term oral memantine treatment, confirming this former study (Figure 14) (Stazi and Wirths, 2021a). Our results are in good agreement with Scholtzowa et al., showing that treated APP/PS1 mice with intra-peritoneal memantine injections of 10 mg/kg for four months revealed a significant improvement in the NOR task (Scholtzowa et al., 2008).

Interestingly, object recognition memory in the NOR seems to be critically dependent on CA1 integrity (Cohen et al., 2013). It has been shown that especially CA1 pyramidal neurons of the distal portion of the CA1 (towards the subiculum) have a crucial role for object recognition (Burke et al., 2011; Masurkar, 2018) and that novel object exposure primarily activates the distal half of CA1 neurons (Ito and Schuman, 2012). Given that the amelioration of neuron loss upon memantine treatment seems to occur mainly in the distal CA1, this might suggest that the rescue in object recognition memory is associated with this hippocampal subfield, which is supported by the correlation analysis (Figure 18B) (Stazi and Wirths, 2021a). As we also detected a positive correlation between neurogenesis rate and NOR performance (Figure 18C) (Stazi and Wirths, 2021a), it is likely that other brain areas such as dentate gyrus or entorhinal cortex (Wilson et al., 2013) also contribute to the rescue of the recognition memory deficit.

Former studies showed that Tg4-42^{hom} mice presented also spatial memory deficits, assessed using the Morris water maze test (MWM). The MWM is a behavioural paradigm for rodents, relying on proximal and distal cues to locate a submerged escape platform. Spatial learning is analysed across repeated trials, including a final probe trial in which the platform is removed from its former position (Vorhees and Williams, 2006).

In addition to beneficial effects on object recognition memory, chronic memantine treatment also improved spatial learning in the MWM in Tg4-42^{hom} mice. Confirming previous data, 6-month-old Tg4-42^{hom} showed a spatial reference memory deficit, indicated by the fact that they did not shown any quadrant preference in the 1-min probe trial (Figure 15C) (Stazi and Wirths, 2021a). In contrast, memantine-treated transgenic-mice spent significantly more time swimming in the target quadrant, indicating an intact spatial reference memory (Figure 15C) (Stazi and Wirths, 2021a).

An amelioration of spatial learning deficits in this task upon memanatine treatment has been shown in various models such as APP/PS1-A246E (Minkeviciene et al., 2004), APP23 (Van

Dam and De Deyn, 2006), or 3xTg (Martinez-Coria et al., 2010; Zhou et al., 2019). In contrast, sub-chronic treatment with daily intra-peritoneal memantine injections of 10 mg/kg failed to rescue memory deficits in 12–15-month-old mice with more advanced pathology but reversed memory deficits in fear-conditioning and spontaneous alternation tasks in young 5xFAD mice at 6–7 months of age (Devi and Ohno, 2016). As for the NOR, some studies report a significant positive correlation between performance in spatial memory tasks and neurogenesis (Ishikawa et al., 2014). While the use of memantine is currently only approved for use in moderate to late AD, our preclinical results might suggest also beneficial effects in earlier disease stages and support thorough evaluation of memantine in early or mild AD.

Taken together the results of the present project support the assumption of a neuroprotective action of memantine since chronic memantine treatment significantly ameliorated CA1 neuron loss and impaired neurogenesis and rescue both recognition and spatial memory deficits of the Tg4-42^{hom}.

4.2. Project II: The effect of long-term oral caffeine intake on the pathology of Tg4-42^{hom} and 5xFAD mice

A variety of previous studies in rodents reported on beneficial effects of chronic oral caffeine consumption with regard to behavioural improvement. In the current subproject, we assessed whether chronic caffeine supplementation in drinking water might exert effects in terms of learning and memory and neuropathological changes in Tg4-42^{hom} and 5xFAD mice. Caffeine supplementation was started at the age of two months and was continued until the age of six months. In the present study, a caffeine concentration of 300 mg/L has been used, which has been shown to result in considerable lower brain and plasma Aβ levels, and restore

cognitive impairments in a variety of previous studies (Arendash et al., 2006, 2009; Laurent et al., 2014).

4.2.1. Part I: Caffeine treatment delays hippocampal neurodegeneration and rescues memory deficits in the Tg4-42^{hom} mouse model of AD

As mentioned in section 4.1, AD patients presented also sensory-motor deficits, so we investigated whether long-term caffeine consumption could help to prevent motor deficits observed in Tg4-42^{hom} mice (Wirths and Bayer, 2010). While there is significant evidence of beneficial effects of caffeine on cognition in different transgenic AD mouse models, only limited data are available regarding its effect on motor function. In the current research project, we observed a partial amelioration of the motor deficits typical of Tg4-42^{hom} mice (Wagner et al., 2019) after chronic caffeine treatment. In fact, in line with a previous study (Almosawi et al., 2018), we did not observed a positive effect of the treatment on the rotarod task (Figure 20A). This finding could be explained by the fact that the effects of caffeine on motor performance appear to be dose-dependent. In fact, low doses of caffeine led to an increased motor function while higher dose decreased it (Abreu et al., 2011; Nikodijević et al., 1993). Almosawi et al. have shown that adolescent male BLC5 mice treated with moderate doses of caffeine (20mg/L) for one week displayed improved motor performance, while mice treated for one week with high doses (200mg/L), so a concentration related to the one that we used (300mg/L), showed the opposite (Almosawi et al., 2018).

While the rotarod is useful to analyse whole motor performance in rodents such as muscle strength, coordination and balance, the disclosure of subtle motor effects demands other tests such as the balance beam task, examining the ability of the animal to stay on a beam. Our results showed that caffeine treatment ameliorates the balance deficit in this task in the Tg4-42^{hom} mice (Figure 20B), a finding being in good agreement with a study reporting that

caffeine alleviates progressive motor deficits in a transgenic mouse model of spinocerebellar ataxia (Gonçalves et al., 2017).

Moreover, we demonstrated that dietary administration of caffeine, beginning at two months of age, to Tg4-42^{hom} mice, provides cognitive protection in both recognition and spatial memory tasks. As mentioned above, neuron loss in the CA1 region of the hippocampus correlates well with memory deficits reported in this mouse model (Antonios et al., 2015; Bouter et al., 2013). Many studies assumed that damage of the medial temporal lobe, including entorhinal, perirhinal and para-hippocampal cortices and hippocampus, causes impairments of recognition memory (Squire and Zola-Morgan, 1991). However, recent evidence supported the idea that the sole injury of the hippocampus area is enough to induce recognition memory deficits (Broadbent et al., 2010; Cohen et al., 2013; Squire et al., 2007).

In this study, recognition memory was assessed using the novel object recognition test (NOR), a widely-used behavioural assay to study recognition memory and object preference in rodents (Antunes and Biala, 2012). At six months of age, Tg4-42^{hom} present with a memory decline in this task (Hüttenrauch et al., 2016a), and this phenotype was completely rescued after long-oral caffeine treatment (Figure 21E and F). Interestingly, our results are in good agreement with studies in which caffeine, similar to us, was used as a pre-treatment to prevent memory damage. In fact, intra-peritoneal caffeine (10mg/kg) administration for four consecutive days prevented the disruption of short- and long-term memory by scopolamine, an anticholinergic drug (Botton et al., 2010). Similar to the work of Botton, another study showed that a single dose of caffeine (10 mg/kg, i.p.) during four consecutive days improved performance of adult CF1 albino mice in the NOR task (Costa et al., 2008).

In addition to recognition memory deficits, Tg4-42^{hom} mice displayed spatial memory decline at six months of age (Antonios et al., 2015). In the current study, long-term spatial

reference memory was assessed using the well-established Morris water maze paradigm (Morris, 1984).

Confirming former studies, spatial memory was impaired in the Tg4-42^{hom} mice, as they did not show a quadrant preference in the 1-min probe trial (Figure 22E). Importantly, this phenotype was completely rescued upon long-oral caffeine treatment, with caffeine-treated transgenic mice performing at WT levels (Figure 22E). This finding is in line with numerous studies in which caffeine treatment results in a reversal of cognitive impairment in different mouse models of AD. One of the first indications supporting a protective effect of caffeine on cognition was provided by Arendash et al. in 2006 (Arendash et al., 2006). In this study, four-month-old APPsw mice received a daily dose of 1.5 mg caffeine until nine months of age, resulting in a significantly improved performance of transgenic animals given caffeine compared to the vehicle group.

As discussed previously, one of the key characteristics of the Tg4-42^{hom} mouse model of AD is the robust pyramidal neuronal loss in the CA1 region of the hippocampus. One of the aims of this project was to evaluate if the rescue of the cognitive deficits after chronic caffeine treatment is supported by an amelioration of the neuronal loss. As in Project I, we analyzed the effect of the caffeine in the two different parts of the hippocampal CA1 area. Interestingly, an analysis of haematoxylin-stained sections revealed a significant amelioration of neuronal loss after caffeine treatment in the distal portion (Figure 23B), pointing again to a strong correlation between the behavioural results and the neuron count analysis (Figure 25A). The current research literature provides some evidence supporting the findings of the present study. In fact, in a rat model of sporadic AD in which neuronal damage was induced by intracerebroventricular streptozotocin administration , caffeine consumption prevented the strepozotocin-induced neuronal loss in the CA1 area (Espinosa et al., 2013). Moreover, caffeine treatment protected nigral dopamine neuron loss in a rat

model of Parkinson's disease (Sonsalla et al., 2012), suggesting more general neuroprotective effects.

In addition to CA1 neuron loss, Tg4-42^{hom} displayed impaired dentate gyrus neurogenesis at six-month of age (Gerberding et al., 2019). New neurons continue to be generated in the sub-ventricular zone (SVZ) and the sub-granular zone (SGZ) of the dentate gyrus of the adult mammalian hippocampus after birth (Encinas et al., 2011; Sorrells et al., 2018). Behavioural studies suggest that adult neurogenesis might have a crucial role in neuroplasticity, neural network maintenance and memory (Christian et al., 2014; Deng et al., 2010; Mu and Gage, 2011). Information regarding the role of caffeine in regulating adult neurogenesis is conflicting. In general, studies have shown that caffeine influences precursor cells proliferation in the DG in a dose-dependent manner (Han et al., 2007; Wentz and Magavi, 2009). In fact, intake of a moderate dose of caffeine (60mg/Kg per day) for seven days, promoted neurogenesis in C57/BL6 mice (Wentz and Magavi, 2009). In contrast, one month of caffeine administration in low doses (0.3 g/L) caused a decline in hippocampus neurogenesis in rats (Han et al., 2007). However, to the best of our knowledge, no study was done showing the effects of caffeine treatment on the prevention of impaired neurogenesis in AD mouse models so far. In the present study, using the same dose of Han et al. but for 4 consecutive months, analysis of doublecortin (DCX)-stained sections revealed that caffeine treatment did not only ameliorate neurogenesis in the Tg4-42^{hom} group, but interestingly also increased neurogenesis in WT (Figure 24). In conclusion, our finding indicates that chronic oral caffeine intake might facilitate neuronal survival. This neuroprotective effect on neuron death well correlate with the overall cognitive amelioration discuss previously.

In summary, based on the results of the present project, it is suggested that in Tg4-42^{hom} AD mouse model, chronic caffeine intake prevents CA1 neuron loss and increases neurogenesis rates and that these beneficial effects are accompanied by a complete rescue of

both recognition and spatial memory however with only a partially amelioration of the motor deficits.

4.2.2. Part II: Oral caffeine treatment has limited effects on the pathology of 5xFAD mice

Recent studies on murine transgenic AD models have claimed caffeine to have therapeutic benefits in terms of reversal of cognitive decline (Arendash et al., 2006, 2009; Cao et al., 2009). Therefore, the aim of this subproject was to examine whether long-term treatment with caffeine was able to restore the spatial memory deficits observed in the 5xFAD mouse model of AD (De Pins et al., 2019), representing a classical model of familial AD with robust extracellular amyloid deposition. Caffeine treatment was initiated at an age of eight weeks, a time point of initial Aβ accumulation in the brain and was continued for four months. The novel object location memory task (NOL) was used to investigate the effects of caffeine treatment on spatial memory. This behavioural assay is based on the ability of mice to recognize when a familiar object has been relocated. Impairment in the NOL task was observed in vehicle-treated-5xFAD groups (Figure 26E), which is consisting with previous findings (Park et al., 2015; De Pins et al., 2019). This impairment was completely rescued in the caffeine-treated 5xFAD mice (Figure 26E and F). Similar results were obtained in former studies using other AD mouse models. In fact, Han and colleagues in 2013, reported that gavage administration of 1.5 mg/day of caffeine, that is exactly the same amount used in our project, was able to completely restore the spatial memory deficits in 12-month-old PS1/APP double transgenic mice (Han et al., 2013). Moreover, Arendash et al. (Arendash et al., 2006, 2009) demonstrated that moderate dose of caffeine (300mg/L) restored working memory in aged cognitively-impaired APPsw mice. Interestingly, these findings are in line with different epidemiological studies suggesting coffee/caffeine as a valid therapeutic for AD. In fact, coffee consumption was directly link to slower cognitive impairments in young as well elderly population (van Gelder et al., 2007; Lorist et al., 1995; Ritchie et al., 2007),

and it was also described that AD patients consumed considerably less caffeine during the twenty years prior the diagnosis (Maia and De Mendonça, 2002).

Recent studies on murine transgenic AD models asserted that the therapeutic benefits on cognition of caffeine are related to reduction of Aβ deposition in the brain (Arendash et al., 2009; Cao et al., 2009). In fact, in APPsw transgenic mice, long-term caffeine administration led to a reduction of both soluble and insoluble Aβ levels in the hippocampus (Arendash et al., 2006, 2009). One of the possible explanations for this finding is decreased Aβ production as a feasible mechanism of caffeine's cognitive protection, due to a reduction in the expression levels of APP processing enzymes such as BACE1 or an up-regulation of enzymes implicated in the non-amyloidogenic processing pathway such as ADAM10.

In this study, however, no reduction in extracellular amyloid plaque load was observed in all the different brain region analysed. Cortex, subiculum, dentate gyrus, and thalamus were evaluated using a pan-Aβ antibody (24311) and a N-terminal specific antibody (82E1) after four month of caffeine treatment (Figure 27A and C respectfully). Differently from the study mention above, we also investigated the potential effect of caffeine on the typical neuroinflammation observed in the 5xFAD mice (Oakley et al., 2006). In line with the results of the plaque load analysis, long-term caffeine treatment did not influence the inflammatory status of the 5xFAD animals (Figure 27E).

In addition, we decided to choose further biochemical assays to support the data observed using immunohistochemistry. Since it is well-established that $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most abundant Aβ variant present in the brains of 5xFAD mice (Wittnam et al., 2012), Aβ levels could furthermore be quantified using ELISA assays. This type of analysis, in comparison to the plaque load quantification by pixel burden, provides a more reliable quantification because Aβ peptide standards are tested alongside the samples to provide standard curves for subsequent analysis of Aβ concentrations rather than relative area percentages. We

analyzed $A\beta_{1-42}$ levels in two brain regions, cortex and hippocampus, and our result confirmed our immunohistochemical data, showing no difference in terms of $A\beta_{1-42}$ levels in caffeine-treated 5xFAD mice (Figure 28).

Since Cao et al. (Cao et al., 2009), proposed caffeine to act on and inhibit the secretases which are involved in APP processing, we focused our analysis deeply on this aspect. A recent *in vitro* study investigating the effects of caffeine and other methylxanthines in APP-transfected SH-SY5Y neuroblastoma cells, also supports such a mechanism and showed decreased Aβ secretion as a function of decreased BACE1 and increased ADAM10 protein levels (Janitschke et al., 2019). In contrast to these observations, Western blot analysis revealed unchanged levels of APP CTFs, APPs total and sAPP-α in caffeine-treated 5xFAD mice, leading to the suggestion that caffeine treatment does not seem to affect APP processing in the current experimental *in vivo* setting. However, it can be postulated that other protective mechanisms are involved in caffeine beneficial effects. For example, Han et al. demonstrated that, chronic caffeine treatment, in PS1/APP double transgenic mice, has a direct link between memory improvements and increasing expression in BDNF and its TrkB (tropomyosin receptor kinase B**)** receptor (Han et al., 2013), both involved in neuroprotection action in the brain (Nagahara et al., 2009). However, the exact mechanism of action of caffeine in our experimental setting in 5xFAD mice requires further investigation.

In summary, based on the results of the present project, it can be stated that long-oral caffeine treatment rescues spatial memory deficits in the 5xFAD mouse model of AD and that this observed beneficial effect of caffeine treatment on memory is independent from changes in Aβ levels, extracellular plaque load or APP processing in the brain of 5xFAD mice.

4.3. PROJECT III: Chronic riluzole treatment restores cognitive deficits, and ameliorates hippocampal neuronal loss and neurogenesis in Tg4-42^{hom} mice

The purpose of this subproject was to evaluate the effect of long-term oral intake of the glutamate release inhibitor riluzole, the only approved drug for the treatment of ALS. Former works evaluated the cognitive decline of the Tg4-42^{hom} mice, utilizing the MWM and the NOR tasks (Antonios et al., 2015; Hüttenrauch et al., 2016a), two of the most widely-used test to study memory impairments in rodents. Hence, it could be demonstrated that chronic treatment with riluzole was able to rescue the recognition memory deficits of Tg4-42^{hom} mice, as the riluzole-treated animals performed at WT levels in the NOR task (Figure 31B and C). Our finding is in good agreement with two studies carried out in Wistar rats, in which systemic administration of riluzole enhanced recognition memory (Sugiyama et al., 2015, 2017). In addition to the rescue of recognition memory, long-term oral riluzole treatment improved spatial reference memory performance of the Tg4-42^{hom} mice as these animals behaved like WT control animals (Figure 32E). Comparable beneficial effects of riluzole on cognition were observed in others rodent models of AD (Mokhtari et al., 2017; Okamoto et al., 2018; Yang et al., 2020).

The behavioural improvements of the Tg4-42^{hom} might be related to reduced hippocampal neuronal loss in the CA1 region of the hippocampus. The connection between hippocampal damage and recognition and spatial memory in rats has been studied previously. Interestingly, spatial memory started to be affected after bilateral dorsal hippocampal lesions that involved 30–50% total volume. In case of larger lesions, not only the spatial memory impairment became worse but also recognition memory started to be damaged (Broadbent et al., 2004, 2010).

Therefore, it can be postulated that a neuronal loss of 50% or even more is critical for an intact hippocampus-dependent cognition. This idea is well supported by Tg4-42^{hom} data in

which the cognitive decline is age and neuronal loss dependent, with memory deficits becoming evident in animals from six- months onwards, showing a reduction of 50% or more in neuron number (Antonios et al., 2015).

In this project, neuronal loss was ameliorated in riluzole- treated Tg4-42^{hom} mice compared to untreated animals (Figure 33). This neuroprotective effect of riluzole in the CA1 region of hippocampus was also reported in other rodents model of AD (Mokhtari et al., 2017), and it was hypothesized that the neuroprotective potential of the drug is due to its ability to attenuate oxidate stress and modulate glutamate levels, that can become, in AD brain, at a toxic concentration (Esposito et al., 2013). In this respect, riluzole was proposed as a glutamate modulator since it can facilitated glutamate re-uptake and decreased its release from the pre-synapsis, regulating the glutamatergic neurotransmission (Azbill et al., 2000; Diao et al., 2013; Fumagalli et al., 2008; Pereira et al., 2017; Dos Santos Frizzo et al., 2004). The effects of long-term riluzole treatment on neurogenesis in AD rodent models are so far unclear. We detected an increase in the neurogenesis rate in the dentate gyrus of the hippocampus after riluzole treatment, with treated Tg4-42^{hom} mice presenting nearly the same number of new-born neurons of their WT littermates (Figure 34). It has been shown that intraperitoneal injection of riluzole (17.5 mg/Kg) enhances the number of new-born neurons in the hippocampus of 6-week-old Sprague-Dawley rats (Topiwala et al., 2017). Therefore, it is likely that both diminished neuronal loss and increased neurogenesis contribute to the beneficial effects observed in riluzole-treated Tg4-42^{hom} mice.

In summary the results of the present project well illustrated that long-term oral riluzole treatment rescues both recognition and spatial memory deficits in six-month-old Tg4-42^{hom} mice. This amelioration in behaviour is accompanied by a decreased CA1 neuron loss and increased neurogenesis in the DG of the hippocampus.

4.4. PROJECT IV: Physical activity and cognitive stimulation ameliorate learning and motor deficits and delay CA1 neuron loss in Tg4-42^{hom} mice

A recent meta-analysis indicates a dose-response link between physical exercise and dementia (Xu et al., 2017), suggesting that physical inactivity might have an important contributing role in increasing dementia risk (Barnes and Yaffe, 2011; Tan et al., 2017). This hypothesis has been extensively investigated in transgenic AD mouse models, in particular using the enrichment environment (EE) paradigm, a well-established setting to study the influence of living in a cognitive and sensory-motor stimulating environment in a standardized way (Nithianantharajah and Hannan, 2006). In contrast to most other AD mouse models, Tg4-42 mice directly express $A\beta_{4-42}$ peptides, one of the most abundant Aβ variants detected in human AD brain (Portelius et al., 2010), in the absence of human mutant APP overexpression. Therefore, the aim of this project was to evaluate the effect of long-term EE housing in a sporadic mouse model of AD.

At the age of 6 months, Tg4-42^{hom} mice displayed clear motor deficits (Wagner et al., 2019) and show decreased physical activity compared to WT mice when housed in a single cage setting (Gerberding et al., 2019). This motor deficit phenotype was completely rescue after 4 month of EE housing, since the Tg4-42^{hom} housed in EE condition display a significant better performance in both rotarod and balance beam tasks respect the SH animals. (Figure 36A and B respectively) (Stazi and Wirths, 2021b), confirming former data in 12-month-old female Tg4-42^{het} mice (Hüttenrauch et al., 2016a). Moreover, increased explorative behaviour and potentially reduced anxiety compared to their SH littermates was observed since Tg4-42^{hom} EE mice spent more time in the central part of the open field (Figure 37B) (Stazi and Wirths, 2021b). Such a phenotype has been also described in 12-month-old WT mice after long-term EE housing (Hüttenrauch et al., 2016b). In line with our findings, it has been reported in rats that living in an enriched environment enhances explorative behaviour (Fernández-Teruel et al., 1997; Widman and Rosellini, 1990) and

activity in general, while on the other hand anxiety-like behaviour is decreased (Modlinska et al., 2019).

To assess working memory deficits in rodents, spontaneous alternation tests like the cross-maze are used. Though we never observed a clear impairment in the in the cross-maze task in the Tg4-42 mice (Hüttenrauch et al., 2016a), as they have an alternation rate score better than chance level (dotted line in Figure 38A) (Stazi and Wirths, 2021b), animals housed under EE displayed a significantly enhanced performance compared to standard-housed animals (Figure 38A), indicating a general beneficial effect of EE living conditions. In this study, we analysed the effect of the living condition also in recognition memory, using the NOR task (Broadbent et al., 2010). Long-term EE living completely rescues the deficits in object recognition memory (Figure 37E and F) (Stazi and Wirths, 2021b) confirming former studies in aged female Tg4-42^{het} mice (Hüttenrauch et al., 2016a). Together with recognition memory impairment, 6-month-old Tg4-42^{hom} SH mice present a spatial reference memory deficit (Antonios et al., 2015; Hüttenrauch et al., 2016a), which can be also completely rescued upon enriched housing living (Figure 39E) (Stazi and Wirths, 2021b). An improvement in spatial learning became already obvious in the cued and acquisition training phases of the MWM task, since the Tg4-42^{hom} EE mice showed an overall significantly better performance than SH indicating a general beneficial effect of housing condition (Figure 39A and C) (Stazi and Wirths, 2021b). These findings are in line with previous data from a completely independent female cohort in Tg4-42^{het} mice (Hüttenrauch et al., 2016a), indicating that the effect of EE housing is not restricted to female mice, as both sexes have been used in the current study. Moreover, we conducted a deep analysis on other MWM parameters, revealing significant effects in a variety of other measures, such as platform or goal quadrant entries (Figure 40) (Stazi and Wirths, 2021b), indicating that the mice housed in EE environment had a complete rescue of spatial memory

deficits (Stazi and Wirths, 2021b). An explanation for this beneficial effects on cognition might be due to the prevention of neuronal loss (Figure 42B) (Stazi and Wirths, 2021b) and increased neurogenesis observed in previously studies using the same mouse model and the same EE paradigm (Hüttenrauch et al., 2016a). The influence of EE has been illustrated in numerous mouse model of AD, mostly those overexpressing a mutant form of human *APP* gene. However, with regard to Aβ pathology, the results of these studies, unfortunately, did not allow universal conclusions since some reported decreased Aβ plaque deposition (Adlard et al., 2005; da Costa Daniele et al., 2020), stable amyloid pathology (Cotel et al., 2012; Francis et al., 2020; Hüttenrauch et al., 2017; Marlatt et al., 2013; Svensson et al., 2020), and even an increased plaque formation (Jankowsky et al., 2003). About this, it is important to note that the improvement in behavioural performance, in this study, has not a direct link between decreasing Aβ levels or plaque deposition as demonstrated in our immunohistochemistry analysis in 6-months-old Tg4-42^{hom} mice upon EE housing (Figure 42A) (Stazi and Wirths, 2021b), 12-month-old female Tg4-42^{het} mice (Hüttenrauch et al., 2016a) as well as in APPsw (Arendash et al., 2004) or APP23 mice (Wolf et al., 2006). Hence, it can be hypothesized that the beneficial effects of living in an enriched environment can be independent from changing in Aβ levels and might be ascribed to the cognitive reserve of the brain that helps counteracting with the neuropathological alteration in AD (Akbaraly et al., 2009; Albert et al., 1995). Therefore, our findings are in line with different studies on the positive effects of EE on cognition in AD mouse models (Dao et al., 2013; Parachikova et al., 2008; Wang et al., 2013). In summary, living in a stimulating environment contribute to ameliorate cognitive deficit in the Tg4-42^{hom}, indicating this kind of preventive therapy as a successful candidate to low the AD risk.

During the MWM evaluation, six of Tg4-42^{hom} SH mice presented a typical floating phenotype and were excluded from the original and classical MWM analysis. Interestingly,

this phenotype was never detected in Tg4-42^{hom} EE mice, indicating a meaningful influence of the housing living condition on this floating behaviour. A similar finding was reported in mice with a targeted point mutation that causes the lack of autophosphorylation at threonine-286 of the α-isoform of Ca2+/calmodulin-dependent kinase II. In particular this mutation leads to spatial learning memory deficit in the MWW and floating phenotype, that were completely rescued after EE housing (Need and Giese, 2003). It was hypothesised that floating behaviour might be connected to a stress condition (Arqué et al., 2008), while EE housing has been involved in decreasing in different stressors factor such as social (Lehmann and Herkenham, 2011) and early life stress (González-Pardo et al., 2019).

Taken together, the results of the present subproject suggest that enriched environment housing rescues sensory-motor impairment and ameliorates cognitive deficits of Tg4-42^{hom} mice. Moreover, long-term enriched living conditions completely rescue the typical floating phenotype of Tg4-42^{hom} mice; however, this beneficial effect on behaviour seems independent from changes in Aβ levels but correlates with decreased neuron loss in the CA1 area of the hippocampus. Our findings are in good agreement with a plethora of epidemiological studies (Iso-Markku et al., 2015; Larson et al., 2006) in which cognitive stimuli during the whole life prevent age-related memory decline and lower AD risk.

5. SUMMARY & CONCLUSION

Nowadays, the only approved drugs to treat AD only act on the symptoms of the disease and they have a poor efficacy on slowing down the progression of neurodegeneration. This could be due to the late administration of the drugs, when the neurodegenerative process is already at a point in which the brain tissue is irremediably lost. Therefore, recent research efforts aim to evaluate preventive and alternative therapeutic strategies to reduce the incidence of the disease.

Therefore, the aim of this work was to investigate the effects of alternative therapeutical approaches on the pathological changes in two different mouse model of Alzheimer's disease: Tg4-42^{hom} and 5xFAD mice.

It is important to note that all the treatment approaches started at 2 months of age so prior to disease onset for both mouse models and continued chronically for four months, employing both genders for all experiments.

The transgenic Tg4-42 model was developed to only overexpress the $A\beta_{4\text{-}42}$ peptide mainly in the hippocampus without any mutations linked to the familial form of the disease. The peptide mainly forms cell-associated neurotoxic aggregates without extracellular Aβ plaque formation. This intraneuronal accumulation of Aβ well correlates with the age- and dose-dependent pyramidal neuron loss in the CA1 region of the hippocampus as well as with robust behavioural deficits. Due to a lack of familiar AD-associated mutations, the Tg4-42 mouse represents a valid model for the sporadic form of the disease.

The 5XFAD mouse is a widely studied and well-characterized model reflecting the characteristics of the familial form of AD. In fact, it is based on the expression of mutant human *APP* and *PSEN1* transgenes, carrying five AD-linked mutations. This model presents an aggressive neurodegeneration with early and fast plaque deposition and behavioural deficits.

In the first three projects, the effects of three pharmacological preventive interventions (memantine, caffeine, and riluzole) were analysed in 6-month-old Tg4-42^{hom} mice. Memantine is one of the drugs approved by the FDA for AD treatment, but its use is authorized only in moderate and severe stages of the disease. Caffeine is the most-widely used psychoactive and CNS stimulant drug worldwide. Different epidemiological studies evidence a positive and preventive effect of caffeine in a variety of diseases, including AD. Therefore, the aim of use these two drugs was to evaluate them effects at an early time point in a sporadic mouse model of AD. Riluzole is the only drug approved for the treatment of ALS and due to its role in the modulation of glutamate release, it represents an interesting candidate as an alternative preventive treatment in AD.

Using a battery of behavioural tests, it was shown that early and chronic treatments with memantine, caffeine or riluzole treatment were able to rescue both recognition and spatial memory deficits, which are typical in Tg4-42^{hom} mice. Motor deficits were only partially rescued, since treated mice showed a better performance only in the balance beam test. Interestingly, these beneficial effects were accompanied by both decreased neuronal loss in the CA1 area of the hippocampus and increased neurogenesis in the DG.

The effects of chronic caffeine treatment were also analysed in the 5xFAD mouse model. Despite the beneficial effect of the treatment on spatial memory, no effects on Aβ levels, plaque deposition, neuroinflammation or APP processing were found in treated 5xFAD mice.

Taken together, the findings of the first three projects support the hypothesis that an early pharmacological intervention has the potential to counteract disease progression in the transgenic Tg4-42^{hom} mouse model of the sporadic form AD. Moreover, despite the beneficial effects of caffeine on the memory deficit in 5xFAD mice, no changes with regard to neuropathology could be detected in this familial mouse model.

Finally, a non-pharmacological preventive intervention employing the well-established EE paradigm was used in Tg4-42^{hom} mice. This paradigm mimics a human stimulating and active lifestyle in mice. Behavioural analyses showed that living in a stimulating environment rescued both memory and motor deficits and that this correlated with a prevention in CA1 hippocampal neuronal death. Interestingly, these effects did not correspond to changes in Aβ levels, in good agreement with the observation in 5xFAD mice treated with caffeine; these two findings might indicate that Aβ pathology is a distinct aspect of the pathology itself.

The current findings demonstrated that cognitive and physical stimulation helped to prevent behavioural deficits and neuronal death in the Tg4-42^{hom} mouse model for sporadic AD.

In conclusion, our findings in two different AD mouse model, strongly argue in favour of the idea that preventive treatments throughout life may be useful as an effective measure to reduce AD risk and the burden of disease progression.

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