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Sledování průběhu patologie podobné Alzheimerově
chorobě v transgenním potkaním modelu TgF344-
AD

**Tracking the progression of Alzheimer's like-
pathology in transgenic rat model TgF344-AD**

Diplomová práce

Vedoucí diplomové práce: Mgr. Petr Telenský, Ph.D.
Praha rok 2022

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne 11.08.2022

.....
Alice Foltýnová

Poděkování:

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Abstrakt

Alzheimerova choroba je progresivní neurodegenerativní onemocnění postihující zejména starší populaci a je nejčastější příčinou demence. Je charakterizované mnoha patologickými událostmi jako jsou amyloidové plaky, neurofibrilární klubíčka, zánět, deregulace neurotransmiterů a mnoho dalších. Tyto disruptivní procesy v konečném důsledku vedou ke ztrátě synapsí a buněk. Podle literatury jsou buněčné ztráty pozorovatelné především v kortexu a hipokampu. Není jasné, v jakém časovém bodě je ztráta buněk signifikantní a v jakých dalších strukturách. Je mnoho dalších důležitých struktur jako jsou čichové laloky nebo entorhinální kortex, kde ztráta buněk nebyla dostatečně kvantifikována. S novými metodami jako je izotropní frakcionace je nyní možné kvantifikovat větší počet struktur mnohem rychleji. Vzhledem k tomu, že není jednoduché získat lidský mozek ihned po smrti, použití transgenních animálních modelů, zejména hlodavčích, je dobrý způsob, jak získat více informací o buněčném a především neuronálním úbytku u Alzheimerovy choroby.

Klíčová slova: Alzheimerova choroba, ztráta buněk, izotropní frakcionátor, hippocampus, neurofibrilární klubíčka, beta amyloid

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting dominantly the elderly and it is the most common cause of dementia. AD is characterized by many pathological events such as amyloid plaques, neurofibrillary tangles, inflammation, neurotransmitter dysregulation and many others. These disruptive processes ultimately lead to synaptic and cell loss which human brain. According to literature, cell loss in AD is apparent especially in the cortex and hippocampus. However, it is unclear at which timepoint the loss becomes significant and in which other structures. There are many other important structures such as olfactory bulbs or entorhinal cortex where the cell loss has not been fully quantified. With new methods like isotropic fractionator it has become possible for scientists to quantify large number of structures more quickly. Since human brains are not easy to obtain shortly after death, use of transgenic animal models, mostly rodents, is a good way to obtain more information about cell and especially neuronal loss occurring in AD.

Key words: Alzheimer's disease, cell loss, isotropic fractionator, hippocampus, neurofibrillary tangles, beta amyloid

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LITERARY OVERVIEW

1. INTRODUCTION

AD is affecting dominantly the elderly population and according to the “Facts and figures” provided by the Alzheimer’s association, it is the sixth leading cause of death in the USA with approximately 5.8 million Americans suffering from it. With our lifespan increasing, the numbers are expected to be more than two times higher in 2050 compared to today. Also, reports between 2000 and 2017 regarding deaths from stroke or heart disease, other major leading causes of death, decrease but deaths caused by AD in fact increase by 145% (Alzheimer’s association, 2019). In addition, existing treatment merely stops the progress of dementia for a while and slightly improves cognitive functioning (Grossberg et al., 2004). All things considered, it is not surprising that AD is one of the most serious health challenges of the 21st century.

1.1. Clinical manifestation of AD

The pathological changes in the brain begin decades before the actual onset of dementia – the post-mortem analysis reveal accumulation of amyloid (senile) plaques (SPs) and neurofibrillary tangles (NFTs) that are further discussed in Chapter 1.2.2. Furthermore, the glucose metabolism decreases 18 years prior to the dementia onset (Mosconi et al., 2008) and eventually the brain starts to atrophy. However, human brain plasticity is remarkable and brain tissue deterioration starts to affect cognition after more than a decade. Because of this, we distinguish 3 stages of AD: preclinical AD, mild cognitive impairment (MCI) due to AD, and dementia due to AD.

Cognitive decline is significant in both MCI and dementia. The important difference is that during MCI stage, the person is still self-sufficient, and the condition can be reversible (Muangpaisan & Petcharat & Srinonprasert, 2012). A meta-analysis showed that 18% of people suffering from MCI return to their previous mental state (Canevelli et al., 2016). In most cases, MCI eventually progresses to dementia. Since the pathological changes are apparent many years prior to the clinical onset of dementia and the boundary between MCI and AD can be considered fuzzy, some scientists prefer the term “prodromal AD” to MCI. According to post-mortem analysis, dementia due to AD consists of 3 stages - namely mild, moderate and severe, each with increasing severity

reflecting the degree of damage to neurons in different areas of the human brain (Braak & Braak & Bohl, 1993).

Dementia is a clinical term represented by a set of symptoms affecting two or more cognitive functions such as memory, language or executive functioning which ultimately lead to incompetence to be left alone and need to have around the clock care (Sandilyan & Denning, 2015). We can distinguish more types of dementia which differ in the pace of cognitive decline and specific clinical manifestations. AD is most commonly represented by a steady and slow cognitive decline (Haxby et al., 1992) with one of the prominent features being the impairment of hippocampus (Allen et al., 2007 ; Sze et al., 1997) which ultimately leads to inability to consolidate any new memories.

However, difficulties with spatial navigation resulting from pathological changes in the entorhinal cortex precede the impaired memory consolidation (Gómez-Isla, 1996) and loss of olfaction (Kovács & Cairns & Lantos, 1999) is one of the first clinical manifestation of AD. After the onset of dementia, the patient goes through the three mentioned stages. As the dementia progresses, the degree of symptom severity increases and new symptoms such as movement disorders, incontinence, undernourishment or neuropsychiatric disorders emerge (Feldman & Woodward, 2005).

It has been observed that there are some ethnic differences in patients with the same type of dementia – e. g., the onset of dementia due to AD is usually 6.8 years earlier for Latino patients compared to Anglo patients (Clark et al., 2005) or from a psychological perspective fewer patients have delusional disorder compared to the majority of AD cases (Craig et al., 2005). Nonetheless, it is safe to say that each type of dementia has its own characteristic set of symptoms. In comparison with vascular dementia (VaD), AD patients tend to be less depressed in the earlier stages and it is possible that they develop severe behavior disorders less frequently (Groves et al., 2000).

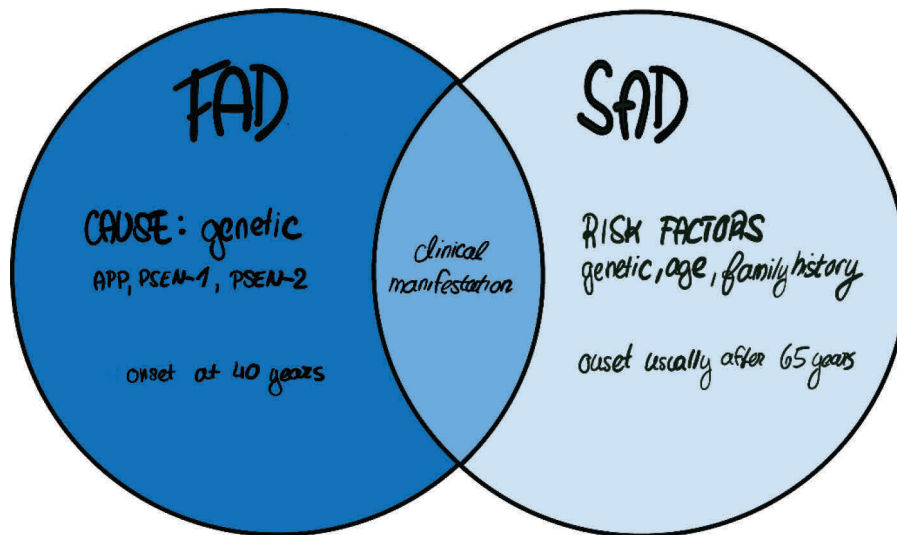


Figure 1. Diagram of differences and commonalities between FAD and SAD. FAD is caused by genetic mutations in APP, PSEN-1 or PSEN-2 whereas the origin of SAD is multifactorial. The most prominent risk factors include APOE4, age and family history. The clinical manifestation is very similar.

As illustrated in Figure 1., we can distinguish two types of AD – familial AD (FAD) and sporadic AD (SAD). FAD is an autosomal dominant form caused by genetic mutations that lead to a higher accumulation of SPs, one of the pathological hallmarks of AD. These are point mutations in amyloid precursor protein (APP), presenilin-1 (PSEN-1) and presenilin-2 (PSEN-2) (Rogaev et al., 1995 ; Schellenberg et al. 1992 ; St George-Hyslop et al., 1987). The symptoms usually arise very early, sometimes even at 40 years of age. (Joshi et al., 2012) However, only a small fraction of AD is considered to be FAD. A meta-analysis revealed that FAD represents 6.9% of AD cases even though it varies in different countries and the previous estimates that proposed a lower number 1-2% are most likely biased (Zhu et al., 2015). The majority of AD cases is SAD and its etiology is considered to be multifactorial. In SAD, genetic predispositions are merely risk factors and not a primary cause. The most significant genetic risk factor is *ApoE4* (Chartier-Harlin et al., 1994) and along with family history (Fratiglioni et al., 1993) and age *ApoE4* falls into the 3 most prominent risk factors of SAD. Despite that, the two types of AD have one thing in common - the clinical manifestation described briefly in the previous paragraph is very similar (Duara et al., 1993).

1.2. Pathophysiology of AD

Multiple risk factors contribute to Alzheimer's dementia, including diabetes (Arvanitakis et al., 2004 ; Su et al., 2019), cardiovascular diseases (Rao & Jackson & Howard, 1999), numerous genetic mutations (Marioni et al., 2018 ; Kretzschmar et al., 2021), stress (Peskind et al., 2001) or brain injury (Mortimer et al., 1991). Decades of experiments provided links between these various pathophysiological events and even though the focus on preclinical dementia and its early stages is very intense, the search for the initial trigger of other pathological events is still ongoing. It is possible that because of the multifactorial nature of SAD that several triggers could initiate the onset and the search for potential disease-modifying agents therefore becomes very difficult.

In this chapter, I would like to summarize the main pathological changes occurring in AD starting with the most discussed pathological hallmarks.

1.2.1. Synaptic and neuronal loss

The malfunction of synaptic transmission is proven to be one of the earliest signs of AD and severely correlates with the cognitive decline to an even greater extent than NFTs (Terry et al., 1991 ; Coleman & Yao, 2003). Cortical neurons have hundreds to thousands of axon terminals and even if a great percentage of them is destroyed, the perikaryon may still survive if the remaining axon terminals still provide neurotrophic factors. However, the cognitive and physiological clinical manifestation starts to show. When a large proportion of presynaptic terminals becomes dysfunctional, the perikaryon is no longer provided with neurotrophic factors, which may induce apoptosis. For this reason, quantification of neuronal loss provides us with important information. The synaptic loss is excessive near SPs (Gonatas & Anderson & Evangelista, 1967) and binding of glutamate to NMDAR (N-methyl-D-aspartate receptor) is necessary for SP induced degradation of synapses (Birnbaum et al., 2015).

1.2.2. AD pathology hallmarks: amyloid and tau

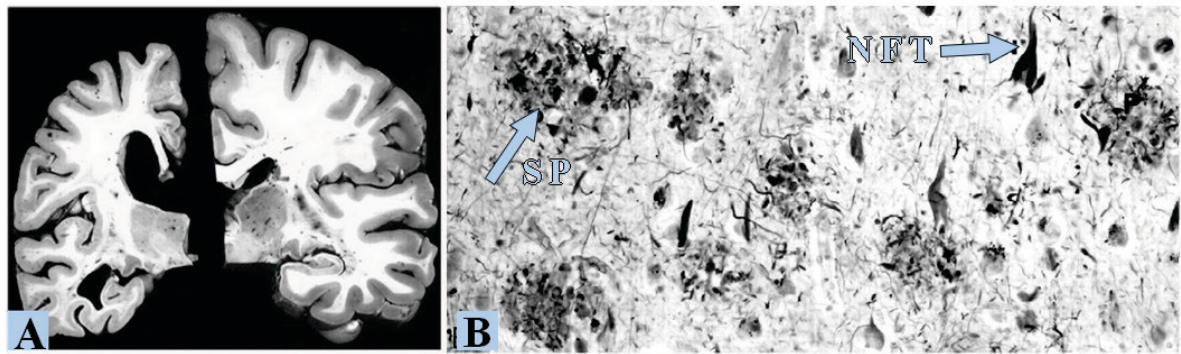
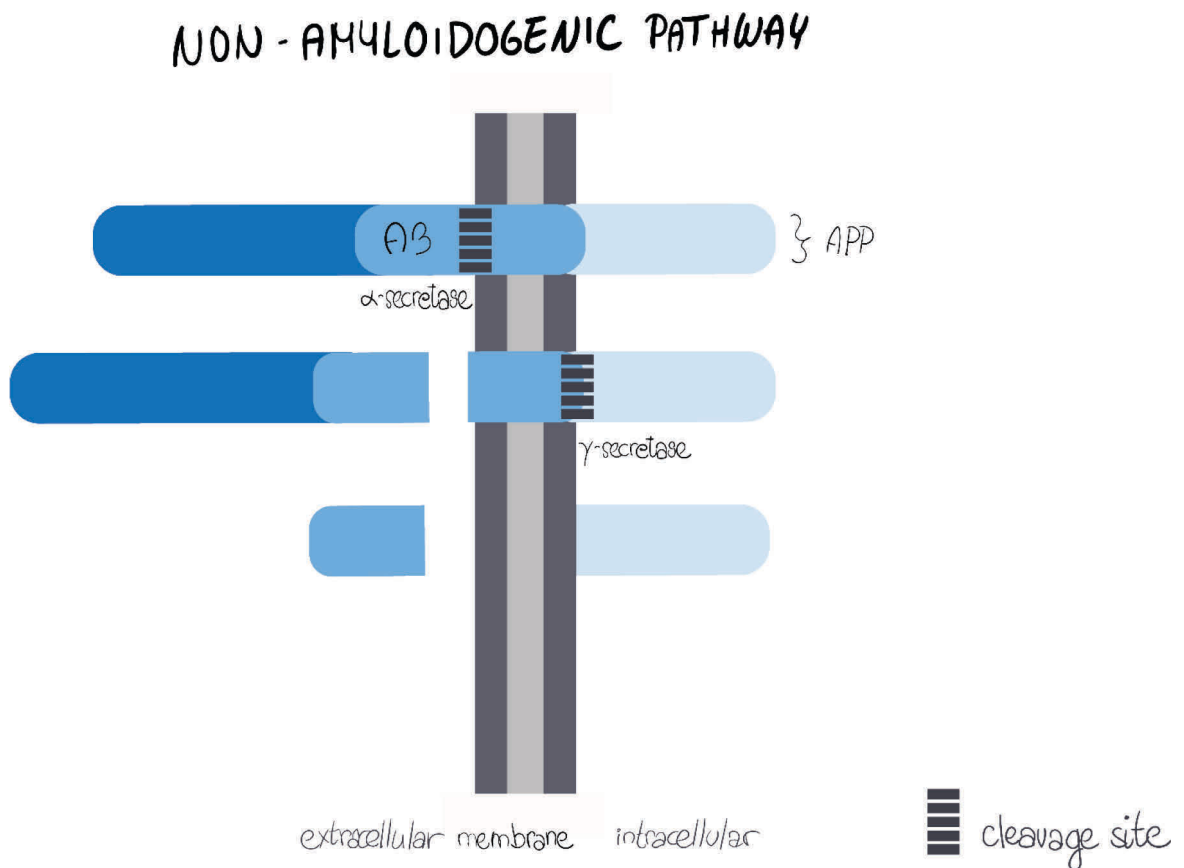


Figure 2. Neurohistopathological changes in AD brain: **A** – on the left is an apparent atrophy of a 70 year old patient with AD and a healthy age-matched control on the right. **B** - Histological image of SPs and NFTs; edited from Wippold et al., 2008.

In 1911, Alois Alzheimer published a paper describing neurohistopathological changes in AD brain and they were named NFTs and SPs (Möller & Graeber, 1998). SPs and NFTs are apparent on a histological image from Figure 2.B. To this day, we consider NFTs and SPs the pathological hallmarks of AD. As shown in Figure 2.A., atrophy in AD brain is very apparent.



AMYLOIDOGENIC PATHWAY

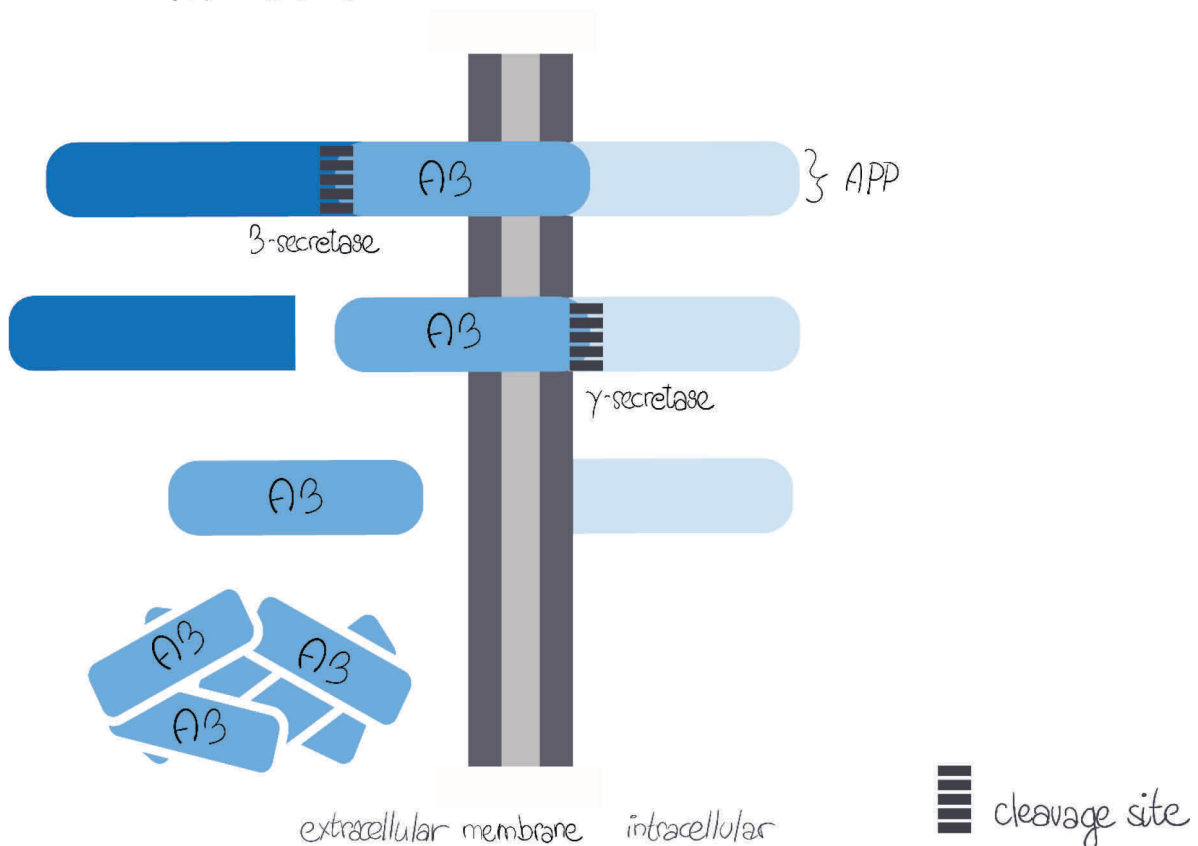


Figure 3. Diagram of non-amyloidogenic and amyloidogenic pathway. APP is cleaved by α -secretase and γ -secretase in the non-amyloidogenic pathway and the remaining APP is then cleaved by β -secretase and γ -secretase which produces A β that accumulates.

As shown in Figure 3., the SPs are a results of beta amyloid (A β) fragments accumulation which are cleaved in the amyloidogenic pathway of a glycoprotein APP. It is a very complex process involving many steps and enzymes. First, the predominant non-amyloidogenic pathway takes place – this process starts with α -secretase cleaving APP (Sisodia, 1992) and the products are soluble and usually not toxic (Lammich et al., 1999). The remaining APP is then cleaved in the amyloidogenic pathway by β -secretase and in both pathways the second cleavage process is enabled by γ -secretase (Vassar et al., 1999).

Even though accumulation of A β is undoubtedly toxic, increased neuronal activity induces A β formation which leads to depression of excitatory synaptic transmission (Kamenetz et al., 2003). There are different forms of A β and A β 42 consisting of 42 peptides tends to be less soluble and accumulates and forms SPs more frequently – the ratio of A β 42 : A β 40 can be used to predict amyloidosis in cognitively normal

individuals who are at risk of AD (Vergallo et al., 2019). Ultimately, the clumps of SPs interfere with neuronal communication. Also, it is probably not only neuron communication that is altered – a study in mouse primary astrocyte cultures showed that A β reduces gap junction communication in astrocytes (Maulik et al., 2020).

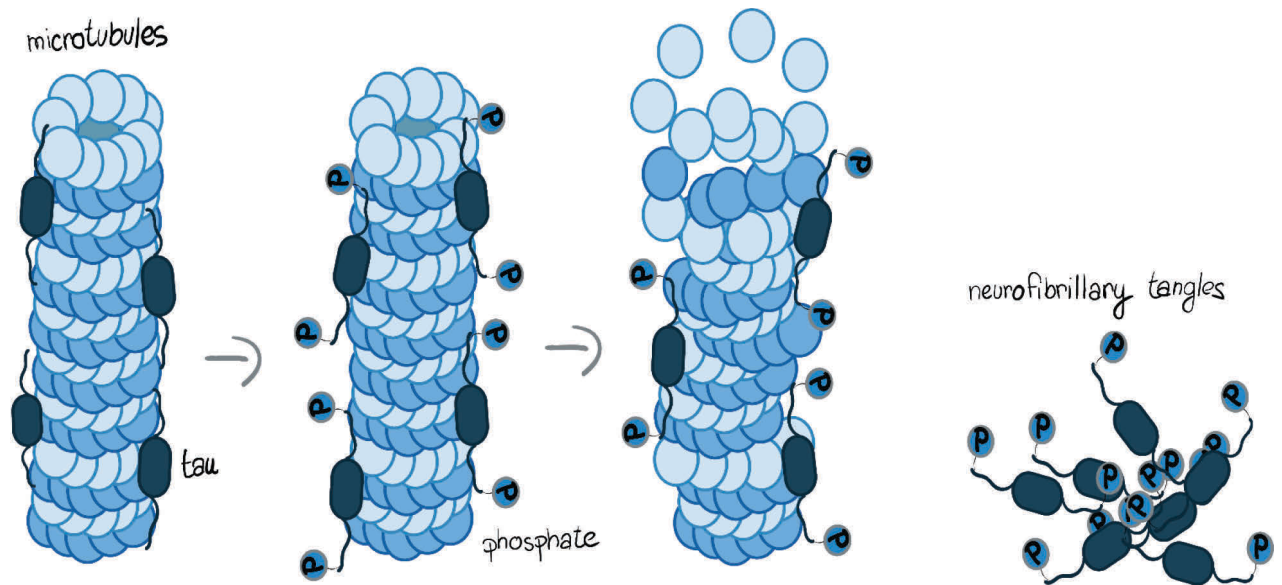


Figure 4. Diagram of NFTs formation. Once the tau protein is hyperphosphorylated, NFTs start to form and microtubules disintegrate.

Tau proteins along with microtubule associated proteins (MAPs) contribute to the integrity of microtubules and neuronal growth. Once the hyperphosphorylation of tau proteins occurs, the integrity is affected so severely that the degradation of cell scaffolding is inevitable. The disintegration of tau proteins forms neurofibrillary tangles (NFTs) as shown in Figure 4., one of the tauopathies characterized by the presence of insoluble tau (Grundke-Iqbal et al., 1986). It takes years for the hyperphosphorylated tau to form NFTs. The formation of NFTs is fastest in the CA1 area of the hippocampus and takes approximately 3.4 years (Bobinski et al., 1998). Authors used criteria provided by Bancher et al. to determine the stage 3 morphology of NFTs where NFTs include both dense aggregates of Tau-1/3–39-positive material, with no discernible nucleus, and clusters of extracellular filaments (Bancher et al., 1989). It is interesting that some brains have a high concentration of NFTs in the temporal lobe without SPs. (Nelson et al., 2009).

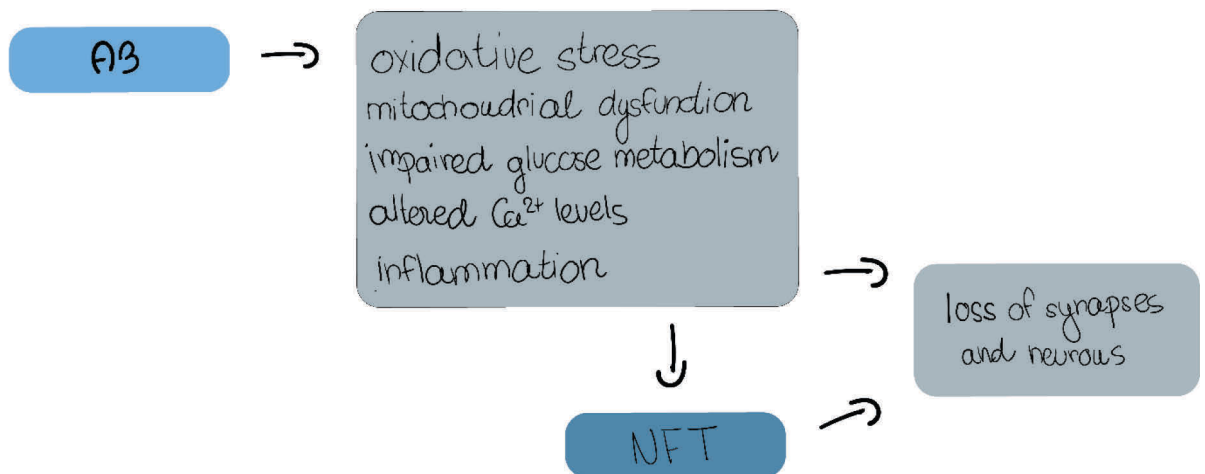


Figure 5. Diagram of ACH . Hypothesis centered around A β as the main trigger of AD. Accumulation of A β leads to various pathological events e.g. oxidative stress and mitochondrial dysfunction, formation of NFTs and neuronal loss.

One of the important questions becomes very apparent – what is the connection between NFTs and SPs and AD pathological events such as inflammation or oxidative stress? In 1992 John A. Hardy and Gerald A. Higgins formed the amyloid cascade hypothesis (ACH) (Hardy & Higgins, 1992) and coined A β as the central AD pathology. The main idea is simple and is summarized in Figure 5. – the accumulation of A β precedes other pathological events and initiates a cascade of disruptive events eventually causing dementia. The ACH theory predicts that the pathology progresses from accumulation of SPs to hyperphosphorylation of tau protein and formation of NFTs. The ACH is strongly supported by the genetic background of FAD since all the mutations contribute to SP accumulation.

Unfortunately, ACH contains many discrepancies. First, there are individuals with increased A β ₄₂ levels who are not demented and sometimes do not even have SPs (Delaere et al., 1990). Additionally, SPs do not correlate with the severity of dementia even though the number of NFTs in fact increases as the dementia progresses. (Arriagada et al., 1992). Importantly, a number of anti-amyloid clinical trials failed (Egan et al., 2018 ; Green et al, 2009 ; Honig et al., 2018). In addition, ACH does not explain why many seniors with SPs do not develop dementia.

For some researchers the discrepancies of ACH discredit the hypothesis but some believe that with certain adjustments the ACH could still be considered a valid hypothesis – e.g. SPs do not correlate with the dementia severity but beta oligomers have been

discovered to correlate with cognitive decline (Tomic et al., 2009). According to some studies even though anti-amyloid treatment is not highly disease-modifying, AD patients could still benefit from it in the initial stages of AD. The Food and Drug Administration (FDA) actually granted an accelerated approval to an anti-amyloid drug called Aduhelm in the USA – a drug containing aducanumab in June 2021 (FDA news release, 2021 ; Dunn & Stein & Cavazzoni, 2021). On the other hand, many controversies surround this approval - not all the data support the claim that Aduhelm administration comes with cognitive amelioration and some even believe that the side effects are stronger than the proposed benefit of the drug (Alexander & Karlawish, 2021).

The question of central role of A β is somewhat controversial (Morris & Clark & Vissel, 2014) and it is important to search for other valid triggers of AD pathology. Some researchers point out that there should be more focus on NFTs, others give credit to viral and bacterial activation of dementia onset focusing more on the inflammation aspect of AD pathology. There are many other theories but we still have a lot to discover before we can fully uncover all the links between SPs, NFTs and other pathological events such as neuronal loss. In the next chapter I would like to elucidate major AD pathological events.

1.2.3. Neurotransmitter dysregulations

Glutamatergic and cholinergic systems are significantly dysregulated in AD. The cholinergic hypothesis of AD proposes that loss of cholinergic neurons in the basal forebrain leads to low levels of acetylcholine (ACh) in cortex and hippocampus (Bartus et al., 1982) and causes cognitive decline as the dementia progresses. The functional neuroanatomy of basal forebrain is further discussed in Chapter 1.3.3. ACh affects processes involved in storage of long-term memory consolidation (Baratti & Opezzo & Kopf, 1993), memory (De Jaeger et al., 2013) and attention (Himmelheber et al., 2000). ACh binds to nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) which are both involved in AD pathology. There is a reduction of binding sites for ACh in AD patients which leads to diminished numbers of nAChRs and mAChRs (Gotti et al., 2006). More specifically, post mortem analysis revealed significantly lower number of $\alpha 4\beta 2$ -nAChRs in AD and a recent PET study showed that this trend starts early on and could potentially be used as an AD biomarker (Sabri et al., 2018). Additionally, acetylcholinesterase (AChE) which is an enzyme responsible for

metabolizing ACh after its release in the synaptic gap is reported to have diminished activity even during MCI (Herholz et al., 2005).

It is important not to overlook glutamatergic dysregulations as well – over 40% of synapses in the brain are glutamatergic (Coyle et al., 1993). Glutamatergic neurotransmission is excitatory. One subtype of glutamatergic receptors are called NMDARs. These receptors are necessary for neuronal survival and plasticity. However, the effect of glutamate after binding to NMDARs varies in different regions of synapses. In case of synaptic NMDARs, cell survival pathways are activated and glutamate binding in the extrasynaptic region results in cell death (Wang et al., 2017). Glutamate levels increase in AD which leads to excitotoxicity (Choi et al., 1988) because it is not possible to keep up the ideal rate of glutamate clearance in the brain. Also, it has been reported that A β directly activates NMDAR (Texidó et al., 2011). NMDAR have a high permeability for Ca²⁺ and perturbations in Ca²⁺ levels inside cells induce neurodegeneration. Glutamate uptake and recycling is severely impaired in AD – there is a severe loss of vesicular glutamate transporter (VGluT) which diminishes transporter activity (Kirvell et al., 2006).

There are attempts to link both these important neurotransmitter systems via a common pathology occurring in AD. Phospholipase A₂ (PLA₂) is decreased in AD and plays role in memory impairment and neurodegeneration by altering both cholinergic and glutamatergic neurotransmission. It appears that PLA₂ is important for APP processing and provides choline for acetylcholine synthesis (Gattaz et al., 1995). NMDARs and A β 42 are able to induce reactive oxygen species (ROS) production in cortical neurons by activating NADPH oxidase. Subsequently, ROS derived from NADPH oxidase activates PLA₂ α phosphorylation which can agitate membrane phospholipids (Shelat et al., 2008).

Dysregulations of glutamatergic and cholinergic systems are often perceived as a molecular target for drug treatment. In Table 1, I summarized current drugs used to ameliorate AD symptoms.

Table 1 – Drugs against AD currently approved by FDA – with the exception of Aduhelm, all treatments used to battle AD are aimed to solve neurotransmitter dysregulations. The drugs approved by FDA in the USA that slow the progress of AD are designed to balance changes in acetylcholine and glutamate in AD.

Drug	Trade name	Mechanism of action	AD stage	FDA approved
<i>Donepezil</i>	Aricept	Acetylcholinesterase inhibitor	All stages	2010
<i>Galantamine</i>	Razadyne	Acetylcholinesterase inhibitor	Mild to moderate	2004
<i>Rivastigmine</i>	Exelon	Acetylcholinesterase inhibitor	Mild to moderate	2000
<i>Memantine</i>	Namenda	NMDAR antagonist	Mild	2003
<i>Donepezil + memantine</i>	Namzaric	Acetylcholinesterase inhibitor + NMDA receptor antagonist	Moderate to severe	2014
<i>Aducanumab</i>	Aduhelm	A β monotherapy	Mild	2021

The majority of approved drugs are acetylcholinesterase inhibitors as shown in Table 1 namely donepezil, galantamine and rivastigmine. Memantine is a NMDAR antagonist and can be prescribed in combination with donepezil. A new drug targeted against A β is called aducanumab.

1.2.4. Impaired calcium signalling

Ca²⁺ acts as a second messenger and transmits external stimuli to respective intracellular targets. There is a well-maintained homeostasis between Ca²⁺ influx and efflux inducing transient fluctuations transmitting to various important signaling pathways. In AD Ca²⁺ homeostasis is disrupted. AD Ca²⁺ hypothesis was formed in 1994 (Khachaturian, 1994).

A β creates pores in the membrane that function as cation channels permeable to Ca²⁺ (Arispe & Pollard & Rojas, 1993) and under specific condition, tau forms these pores as well (Esteras et al., 2021). The higher intercellular levels of calcium then accelerate the A β accumulation and beta oligomers negatively affect calcium levels ultimately creating a toxic loop leading to the inability of cells to put calcium levels in order and inevitable apoptosis.

AD associated neurotoxicity has been previously linked to calcium binding proteins. Neurons containing parvalbumin appear resistant to ischemia and their numbers are diminished in AD (Zallo et al., 2018).

1.2.5. Metabolic dysregulations and oxidative stress

Another prominent category of AD pathology falls into the spectrum of metabolic dysregulations which contains a large number of pathologic alterations and some of them can be potentially used as biomarkers – e.g. according to a metabolomics analysis, sphingomyelins and phosphatidylcholines are altered in the preclinical stages of AD (Toledo et al., 2017). AD in ApoE- ϵ 4 carriers worsens degradation of lipids in blood (Keller et al., 2011).

AD is characterized by changes in expression of genes involved in glycolytic metabolism. Glucose hypometabolism appears early on in AD development and is a good indicator of possible decline from MCI to AD in APOE4 carriers (Mosconi et al., 2004). On the hormonal level, this condition is caused by insulin malfunction. One of the physiological roles of insulin in brain, muscle and adipose tissues is to increase glucose uptake and store energy. After insulin binds to its surface receptors, GLUT4 (glucose transporters 4) translocate to the surface within minutes and enable glucose uptake. (Bryant & Gould, 2011). If normal doses of insulin are not sufficient for GLUT4 translocation, i.e. (that is) insulin resistance, diabetes mellitus and other conditions can develop. Importantly, the insulin resistance interlinks AD pathology with pathology of diabetes mellitus I and II leading to some researchers claiming that AD is a diabetes type III. (Steen et al., 2005).

Another implication of glucose hypometabolism is a downregulated acetylcholine synthesis. The impaired glycolytic pathway does not produce enough acetylcoenzyme A, key component of acetylcholine synthesis (Rivera et al., 2005).

The decreased levels of insulin in AD lead to decreased choline uptake activity. A study in SH-SY5Y cells showed that low levels of insulin cannot regulate the activity of the high-affinity choline transporter (CHT) properly (Fishwick & Rylett, 2015).

Apart from insulin, other hormones important for metabolism such as vitamin D could contribute to AD if not in perfect homeostasis. Lack of vitamin D creates a perfect environment for AD pathology and disturbs phagocytation of SPs. (Mizwicki et al., 2012). Additionally, the lack of vitamin D also worsens absorption of calcium (Yoshizawa et al., 1997). Another example is glycogen. Astrocyte reserves of glycogen represent the majority of brain glycogen. Glycogen breaks down to lactate which benefits glutamatergic neurotransmission and synaptic plasticity. In AD, glycogen synthase kinase

3 (GSK-3) is upregulated which leads to diminished glycogen synthesis. Inhibition of GSK-3 leads to cognitive improvement in A β 42 oligomer infused rats but the effects could be harmful in healthy controls (Begum et al., 2009). Glycogen and its degradation also plays a role in memory formation (Duran et al., 2013). Degradation of glycogen is also disrupted in AD, a study showed that A β 42 oligomers reduce concentration of α -amylase that degrades neuronal glycogen in CA1 of hippocampus which is important for memory formation (Byman et al., 2021).

Oxidative stress is defined as an imbalance between the ROS and ROS removal by antioxidants. It has been proposed as another pathology contributing to early AD pathology and ultimately cell loss. Mitochondrial and metal abnormalities may be crucial in indicating oxidative stress in AD. Mitochondrial DNA in AD exhibits greater fragmentation and reduced antioxidant levels among other alterations (de la Monte et al., 2000). Also, the markers for oxidative stress (e.g. oxidatively modified products of nucleic acids and proteins, products of lipid peroxidation) have been observed early on in MCI stage (Butterfield et al., 2006) and more markers appear as dementia progresses.

1.2.6. Inflammation

The supporters of ACH believed that A β accumulation leads to activation of microglia resulting in inflammation mediators release such as cytokines. A β does contribute to microglial activation but it also appears that microglia are activated before SPs accumulate between neurons. Microglia release free radicals that attribute to A β accumulation but they also clear SPs by fagocytation and lower concentration of A β . (Ewers et al., 2020). This is the beginning of a vicious circle that becomes uncontrollable and destructive in AD. The importance of studying microglia in relation to neurodegeneration has been highlighted in the last few years because genome wide association study (GWAS) found that a lot of genes that present a risk factor in developing AD are expressed in microglia (Wightman et al., 2020).

Astrocytes also contribute to clearance of A β in an ApoE dependent manner (Arélin et al., 2002) and alter inflammation by restricting its spread throughout the brain. This is enabled by perivascular spaces (PVS) transport. Macromolecules and solutes can enter CNS via PVS which represent a thin region between cells and surround blood vessels that supply blood to CNS. Perivascular spaces communicate with cerebrospinal

fluid so that the transport is possible in both directions. In AD, A β is deposited in PVS in walls of capillaries and arteries as well as the extracellular spaces of the cerebral cortex (Preston et al., 2003). On the other hand, in AD, astrocytes can become less efficient in their metabolic roles and can even release neurotoxins. Impaired astrocytic clearance contributes to cognitive impairment even in normal aging (Kress et al., 2014).

In this chapter I merely touched upon basic AD pathophysiology and summarized the prominent dysregulations that lead to neuronal loss and are therefore crucial for my master's project. There are many other aspects worth mentioning e.g. – some viral and bacterial infections can contribute to pathological inflammation and the dementia onset or the accumulation of iron that occurs in AD can create electronic noise background and interfere with neuronal communication (Kletetschka et al., 2021). Furthermore, since 1993, vascular AD hypothesis (De la Torre & Mussivan, 1993) which centers around brain hypoperfusion in AD exists even though it did not receive as much attention as only a year older ACH did – and perhaps unfairly. Additionally, the postmortem analyses revealed that it is very common to have mixed dementia – 60-70% cases of AD also exhibit vascular dementia pathology (Alzheimer's association, 2019).

1.3. Structures susceptible to AD

To understand susceptibility of individual brain regions to AD, it is important to compare changes in AD brains with normal aging. In contrast to dementia, multiple stereological human analyses showed that the number of neurons in human brain does not decrease with normal aging even though the morphology could change and neurons appear smaller (Haug et al., 1984 ; Gómez-Isla et al., 1996). However, a study reported a localized cell loss in CA1 part of the hippocampus. (West & Gundersen, 1990) but the cell loss is not general. A small but significant decrease of neurons has been observed in amygdala (Herzog & Kemper 1980). However, in a newer study, decrease of neurons was not significant and the volume of amygdala did not change either (García-Amado & Prensa, 2012). In primates, electrophysiological properties of neurons change even in normal aging and synaptic excitation is decreased (Luebke et al., 2004) and there is synaptic loss in prefrontal cortex (PFC) (Peters & Sethares, & Luebke 2008). Synaptic loss is apparent in some regions in human studies as well e.g. precentral gyrus (Adams, 1987) and neocortex (Masliah et al., 1993). Conversely, a newer study reported that

there is no significant loss in PFC in normal aging suggesting that synaptic loss is a result of neurodegeneration (Scheff et al., 2001).

Multiplex network system analysis that describes topological organization of brain connectivity revealed functional networks in AD brains to be more heterogeneous compared to controls. Hippocampus, occipital areas and posterior default mode network (DMN) regions including temporal lobe, PFC, cingulate cortex and parietal cortex seem to be more susceptible to neurodegeneration in AD (Yu et al., 2017). These findings correlate with cognitive decline and accumulation of A β (Klunk et al., 2004).

It is important to quantify how the selective vulnerability reflects in the changes of neuronal numbers, synaptic loss and the presence of AD pathology hallmarks. The data on this matter are incomplete and comparisons between studies is difficult due to limited number of structures of interest – typically just hippocampus and neocortex. In addition, many studies include only one timepoint or when multiple timepoints are used, the timescales are often inconsistent. Also, more research is needed to determine the changes in glial cell numbers since most studies focused exclusively on neurons.

Different neuronal subtypes can vary in the vulnerability to neurodegeneration in AD and this selective vulnerability is a fundamental feature of neurodegenerative diseases. Large pyramidal neurons with long axons appear to be more affected than smaller neurons. This could be due to the fact that NFTs tend to accumulate rather in the pyramidal neurons (Mann & Yates & Marcyniuk, 1985 ; Merino-Serrais et al., 2013). Due to their rather large cell surface, they are more likely to be affected by toxic environment caused by AD. Pyramidal neurons also have high metabolic rates which means that they are very sensitive to glucose hypometabolism. Importantly, these neurons form input and output pathways to the association cortex and their loss contributes to the cerebral atrophy characteristic for AD.

1.3.1. Olfactory bulbs

The olfactory bulbs (OB) are a part of the forebrain responsible for olfaction (Shepherd & Greer, 1998). In adults, deficient olfaction is linked to higher long-term mortality. There are many factors contributing to this association such as safety issues e.g. food poisoning or gas leaks and also neurodegeneration (Devanand et al., 2015 ; Liu et al., 2019). The impaired sense of smell can be used as an early AD biomarker (Velayudhan et al., 2013) since it is one of the first prominent clinical features of AD.

Interestingly, combination of *ApoE4* and impaired olfaction leads to a decline in cognitive function even in non-demented individuals (Graves et al., 1999). As a result of AD in humans, volume and neuronal numbers in OB are decreased (Ter Laak & Renkawek & Van Workum, 1994) and there is a loss of dopamine receptors (Loopuijt & Sebens, 1990). It is important to find which pathologic alterations precede the pathological hallmarks of AD and how the pathological hallmarks interfere with OB function. In APP/PSEN-1 mice, certain signaling pathways such as MAPK/ERK important for cell proliferation are dysregulated before A β accumulation in OB (Lachén-Montes et al., 2016). Another finding in rats suggests that A β oligomers interfere with olfactory information output (Hu & Geng & Hou, 2017).

1.3.2. Hippocampus

Hippocampus is located in the medial temporal lobe and has been thoroughly studied since the 1950s for its well-defined structure and a key role in memory and learning. The changes in hippocampal volume are significant very early in the course of AD pathology, and can predict the risk of conversion from MCI to AD (Jack et al., 1999 ; Leung et al., 2010). Synaptic loss becomes apparent even during the MCI stage (Scheff et al., 2006). In line with the disrupted calcium levels in AD, there are reports on the selective loss of parvalbumin positive interneurons in CA1 of hippocampus of 3x Tg mice (Zallo et al., 2018).

1.3.3. Basal forebrain

Basal forebrain (BF) structures are located in the rostral part of the brain called forebrain to the front of and below the striatum. BF consists of important cholinergic nuclei e.g. ventral basal ganglia (nc accumbens, ventral pallidum), nucleus basalis, diagonal band of Broca, substantia innominata, and the medial septal nucleus. BF is crucial in regulating sleep activity. Acetylcholine produced by cholinergic neurons is important for wakefulness and is very high during REM sleep (Vazquez et al., 2001). Since body temperature strongly affects quantity and quality of sleep, BF also plays role in its regulation (Kumar & Kumar & Mallick, 2011) and thermoception. BF has important connections to the neocortex and hippocampus, the majority of cholinergic innervation to the hippocampus and neocortex is provided by BF neurons. Compared to

neocortex, BF shows signs of greater neurofibrillary neurodegeneration in AD patients (Cullen & Halliday, 1998). Neuronal loss has been reported in important cholinergic and aminergic nuclei since decreased cholinergic tone is one of the prominent AD pathologies. In humans, the loss is apparent in the nucleus basalis (Whitehouse et al., 1981) which contains the most acetylcholine neurons.

1.3.4. Neocortex

Neocortex in mammals consists of six layers and in humans it represents the largest part of the cerebrum. It underlies the highest order functions including cognition and motor commands. The atrophy of the neocortex is apparent in the MCI stage and starts in the medial temporal, middle and inferior lateral temporal, inferior parietal, and posterior cingulate cortex and then progresses to parietal, frontal, and lateral occipital cortex, followed by anterior cingulate cortex (McDonald et al., 2009). Diminished number of neurons in cerebral cortex is a long known pathology (Kobayashi & Emson & Mountjoy, 1989) in AD. Synaptic loss in the PFC correlates with cognitive dysfunction (DeKosky & Scheff, 1990) and there is a decline in numerical synaptic density in neocortex (Scheff & Price, 2006).

Important part of neocortex is the entorhinal cortex (EC) located in the medial temporal lobe. It is the main input and output structure connecting the hippocampus with the cortex, and contributes to memory formation, navigation and time perception. The chronic inflammation typical for AD contributes significantly to cell loss in EC in TgF-344 AD rats (Hauss-Wegrzyniak et al., 2002). In humans, the loss of neurons in EC starts early during the MCI stage (Kordower et al., 2001).

1.3.5. Cerebellum

Cerebellum is a part of rhombencephalon and is important for motor control but also cognition. Cerebellum seems to be relatively resistant to some degree in AD – fewer apoptotic pathways are activated (Xu et al., 2019). However, the volume of cerebellum in AD patients is decreased by 12.7 % but neurons numbers are mostly preserved (Andersen & Andersen & Pakkenberg, 2012).

1.4. Animal models of AD

Nonetheless, in animal models, scientists developed multiple approaches to achieve AD-like pathology. The pharmacological models cause imbalances in neurotransmitter levels mimicking SAD etiology and many transgenic models are caused by mutations that lead to higher accumulation of A β and thereby reflect the origin of FAD more realistically than SAD.

The pharmacological models often use injection of scopolamine (Bajo et al., 2015). Scopolamine is a parasympatholytic known for its antagonist effects on muscarinic receptors. After administration, the levels of acetylcholine are decreased in concordance with AD pathology which mimics the clinical AD symptoms to a certain degree. Cholinergic depletion compromised attention tasks but memory consolidation is not as severely impaired as in AD (Kopelman & Corn 1988). Another option is to affect glutamatergic system using NMDA receptor antagonist called ketamine (Yamakura et al., 1993). The main issue with pharmacological models lies in their inability to reproduce AD pathophysiology in a more complex way. Combination of drugs could in theory have a cumulative effect and cause a more diverse AD-like profile. A number of studies combined scopolamine with such agents but the results were not significant or the effect was minimal (Broocks et al., 1998 ; Vitiello et al., 1997). On the other hand, combination of scopolamine and NMDA antagonists potentiates scopolamine-induced memory impairment in rats (Li et al., 1997) but we lack data in humans.

Another option is an intracerebroventricular application of streptozotocin (STZ). STZ represents a neurometabolic model that mimics SAD etiology, the cholinergic depletion is very apparent as well as oxidative stress and glucose hypometabolism. It is a betacytotoxic drug causing DNA alkylation. Contrary to the pharmacological models, STZ induced changes are irreversible (Kamat, 2015).

Scientists have developed multiple transgenic animal models of AD. For large scale and gene disruption experiments transgenic *Drosophila* (Iijima & Gatt & Iijima-Ando, 2010) or *C. elegans* (Link et al., 2003). AD models are suitable. For more complex behavioral and histological assays we have mouse and rat models of AD. There are some advantages in choosing rats as an experimental model. Rats share more similar genetic and physiological characteristics with humans compared to mice (Gibbs &

Pachter, 2004 ; Jacob & Kwitek, 2002). Also, rat brains are bigger in comparison with mice which can be advantageous in detailed anatomical studies where the yield of tissue is a critical factor.

1.4.1. TgF-344 AD model

In my project, a transgenic rat model TgF-344 AD has been used. This transgenic strain is based on Fisher F344 rat strain, which is naturally susceptible to neurodegeneration and considered a model of accelerated aging. In TgF344-AD rats, human APP with the swedish mutation and human PSEN-1 with the Δ exon 9 mutation are expressed under the mouse prion promotor (Cohen et al. 2013). One of the advantages of this model is that the NFTs and SPs are first apparent around 6 months and the accumulation is age-dependent. Also, this model exhibits altered cortical and hippocampal excitability previously not described in other transgenic AD models (Stoiljkovic et al., 2018) and there is apparent functional hypoconnectivity as an early sign of neurodegeneration (Anckaerts et al., 2019).

The mutations in TgF-344 AD are the same that cause FAD in humans. Point mutations are induced in human amyloid precursor protein (APP) which is a Swedish mutation and human presenilin-1. The mouse prion promoter is used for both transgenes (Cohen et al., 2013).

Multiple behavioral assays were performed on TgF-344 AD rats as well and researchers found that these rats exhibit signs of anxiety early-on (Pentkowski et al., 2018), they suffer from hyposmia (Sar   et al., 2020) and learning deficits (Tournier et al., 2021).

Also, a study observed that even though retinal ganglion cell number does not differ, TgF-344 AD have hypertrophic retinal pigment epithelial cells and visual acuity was lower (Tsai et al., 2014) which is in concordance with visual dysfunction that contributes to visual impairment in humans (Rizzo et al., 2000).

1.5. Comparison of methods used to quantify neurons

For 150 years scientists have tried to quantify neurons. Nonetheless, some of the original assumptions about neuronal counts presented in textbooks were inaccurate to say the least and in some cases are impossible to support with relevant research. This

is due to the fact that the early attempts to establish neuronal counts contended with many difficulties – e.g. low resolution of microscopes and fixation and staining techniques needed to be improved (Gittins & Harrison, 2004). The methodology was inconsistent until the principles of stereology were developed. Until then, scientists believed that the lower density of old people brains correlates with lower number of neurons even in brains with no signs of neurodegeneration (Brody, 1955).

1.5.1. Stereology

All methods of cell quantification fundamentally count cell bodies with its nuclei. The stereological analysis is the most common used method and was first described in the 1940s (Abercrombie, 1946), although it is time consuming and can be expensive. Stereology is originally a three-dimensional interpretation of observation of two-dimensional cross sections of materials or tissues. It can be model-based (Baddeley & Gundersen & Cruz-Orive, 1986) or design-based (Schmitz & Hof, 2005). Each comes with different pros and cons – nonetheless, the neuron number estimates obtained from these two approaches do not significantly differ (Baquet et al., 2009). In the model based approach, the cells are counted in thin sections and the numbers are extrapolated for the sections that are not counted. Correction factors are applied to account for the fact that larger cells can be seen in more than one section. The design-based approach works with thicker sections. Sections are selected randomly and the schematic distribution of cells in counted sections is applied to the entire reference space. In theory, we can consider this assumption unbiased but changes during the tissue processing such as deformation and cell loss need to be taken into consideration. As the design-based approach suggests, probes and sampling schemes are designed a priori so that size, shape and spatial orientation of cells is not taken into consideration.

1.5.2. DNA extraction

The second approach is DNA extraction and measuring DNA content based on knowledge of DNA content per cell nucleus. (Heller & Elliott, 1954) However, this method is susceptible to contamination and cannot differentiate specific cell types. Nonetheless, it is worth noting that this method has been proved as valid as stereology

and also as the newest approach in counting cell estimates – isotropic fractionator (IF) (Bahney & von Bartheld, 2014).

1.5.3. Isotropic fractionator

IF has been pioneered by S. Herculano-Houzel (Herculano-Houzel & Lent, 2005). This method is a more time efficient technique compared to stereology. The individual structures are homogenized manually using a detergent in order to penetrate the cell but leave the nucleus intact. The nuclei are then visualized by DAPI. The cell estimates can be counted either manually using a Neubauer chamber or the method can be even faster by employing flow cytometry (Collins et al., 2010). Because of a large number of structures that are quantified in my master's project, IF is the most suitable method. One of the important contributions of IF is that many scientists started to focus on neuron to glial cell ratio and it became finally clear that glial cells do not outnumber neurons and the ratio is close to 1:1 (Azevedo et al, 2009).

EXPERIMENTAL PART

2. AIMS OF MY THESIS

The studies that establish neuronal changes in AD usually focus on a limited number of structures, e.g. hippocampus or neocortex. Compared to stereological methods, IF provides a less time-consuming approach and enables to conduct a more comprehensive study focused on a high number of structures. The main aim of my master's project was to quantify changes in neuronal and nonneuronal cells as well as their densities in 6, 12 and 19 months-old TgF-344 AD rats and aged-matched wildtype controls in 14 structures of the rat brain: olfactory bulbs, olfactory cortex, prefrontal cortex, basal ganglia, diencephalon, mesencephalon, dorsal hippocampus, ventral hippocampus, entorhinal cortex, pre/postsubiculum, retrosplenial cortex, rest of cortex. The second aim was to examine selective neuronal vulnerability pattern in TgF-344 AD and establish whether the same structures affected early-on in human AD overlap in TgF-344 AD.

3. MATERIALS

3.1. Animals

In this experiment, TgF344-AD male rats and wildtype male rats from strain F344 (WT) were used. The animals were taken care in line with accepted standards. They were placed in boxes with 2-4 rats, in 12/12 day/night régime (the lights were turned on at 7:00 each day) and the room temperature was 22 ± 2 °C. All rats could eat and drink at any time of the day.

3.2. Instruments and devices

Analytical weighing machine, Micro precision calibration, USA
Binocular, Olympus SZX10, Japan
Centrifuge, Hettich, Germany
Eppendorf tubes 500 µl, 2 ml, 10 ml, 20 ml, Sigma-Aldrich, Germany
Fluorescent microscope, Olympus, Germany
Lauben sous vide (water bath), Germany
Mechanical homogenizer, China
Microwave oven Gallet galfmom 420W, France
Multiwell plate, Sigma-Aldrich, Germany
Neubauer chamber, Sigma-Aldrich, Germany
Pipettes, Eppendorf, Germany
Plastic pipettes, ThermoFisher, USA
Surgical instruments (microknife, tweezers), Sigma-Aldrich, Germany
Syringe, ThermoFisher, USA
Vibrotom Leica, Germany

3.2.1. Chemicals

Agarose, Sigma – Aldrich, USA
DAPI, Sigma-Aldrich, USA
Dihydrate sodium citrate, Sigma-Aldrich, USA
Ethanol, Serva, Germany

Glycerol, Sigma-Aldrich, USA
Heparin, ThermoFisher, USA
NaCl, Carl Roth, Germany
Triton X-100, ThermoFisher, USA

3.2.2. Solutions

Antifreeze - 30% glycerin, 30% ethylene glycol, 10% 0.25M PBS, 10% H₂O
DS – 1% Triton, 90% H₂O, 1.7 g dihydrate sodium citrate
PBS-TX – 0,5% Triton X-100, 99% 0.25M PBS

3.2.3. Antibodies

Primary: anti-Neun (rabbit), Sigma-Aldrich
Secondary: Alexa Fluor™ 594, (goat, anti-rabbit)

3.2.4. Software

GraphPad Prism 5.03 (GraphPad Software Inc, California, USA, www.graphpad.com)
Statistics Calculator Pro 3.0.2. (free software, www.apkpure.com/statistics-calculator-pro/com.cgollner.pro)

4. METHODS

6 and 19 months old brains of TgF-344 AD (n=5,7) were collected with age-matched wildtypes (n=4,8) and processed using IF to quantify neuronal and cell numbers and densities.

4.1. Isotropic fractionator

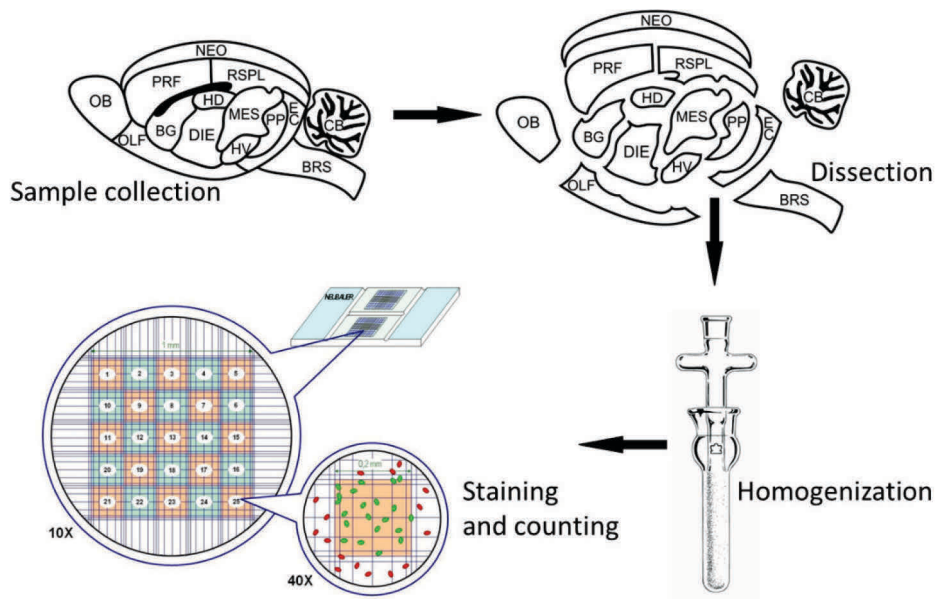


Figure 7. Diagram of IF. The rat brain is dissected into 14 structures, each structure is homogenized and then cells and neurons are quantified under a fluorescent microscope using Neubauer counting chamber.

In Figure 7., diagram of IF describes methodology divided into 4 steps– sample collection and preparation, dissection, homogenization and staining and the fourth one – staining and counting.

4.1.1. Sample collection and tissue preparation

4.1.1.1. Tissue preparation: Perfusion

In total, 24 brains were collected, perfused and fixed in 4% paraformaldehyde (PFA). First, animals were anesthetized with ketamine and xylazine (1:3, 0.25ml/100g of ketamine, 0.75ml/100g of xylazine). Once they were under anaesthesia and did not flinch while pinching the tail, perfusion could start. First, a cut below sternum along both sides of the ribs was performed in order to open the chest cavity. The ribs were

either cut off or pinned down by surgical clamps to secure a good approach to the heart. Heparin injection (approximately 500 U for an adult rat was induced with a syringe to prevent blood clots). A needle with a cannula was then induced inside aorta and left ventricle was cut open. First, the blood circulation was washed with PBS for approximately 3 minutes to wash away any blood from the brain. Then, the blood circulation was washed with 4% PFA for approximately 5 minutes to fixate the brain tissue. The brain was then dissected as a whole and placed in small bottles filled with 4% PFA and post-fixed for 12 hours. Then, the brain was permanently placed in AF and stored in the freezer at -20°C .

4.1.1.2. Tissue preparation: Vibratome

In order to make 300 μm sections, brains were soaked in PBS for 12 hours prior to using vibratome. Then, they were sliced in half with a razor blade and placed in agarose. Subsequently, each half was cut in two homogenous blocks. The blocks were glued to the vibratome and cut to 300 μm sections and placed in a small bottle filled with PBS.

4.1.2. Dissection

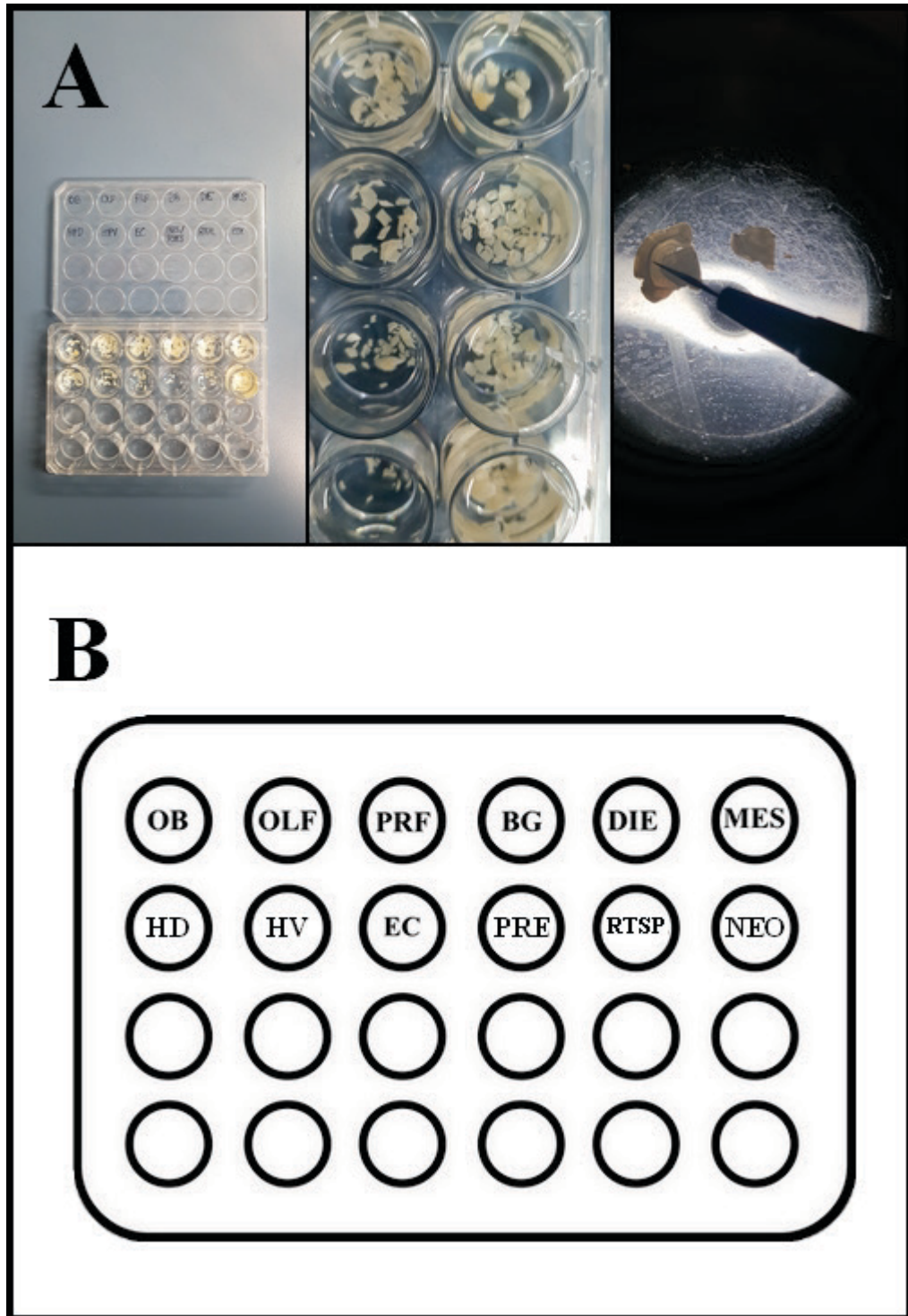


Figure 8. Diagram of microdissection. **A:** photo documentation of microdissected brain **B:** illustrated diagram of multiwell plate with collected structures.

In Figure 8., the processing of 300 μm sections during microdissection is explained. The sections were placed in a Petri dish filled with PBS. Then, the sections

were split into 12 structures with a surgical microknife and placed into 12 wells of a multiwell plate using tweezers.

The structures were: OB (olfactory bulbs), OLF (olfactory cortex), PRF (prefrontal cortex), BG (basal ganglia), DIE (diencephalon), MES (mesencephalon), HD (dorsal hippocampus), HV (ventral hippocampus), EC (entorhinal cortex), PRE (presubiculum), RTSP (retrosplenial cortex), NEO (rest of cortex). The CB (cerebellum) and BSTEM (brainstem) were dissected with a scalpel.

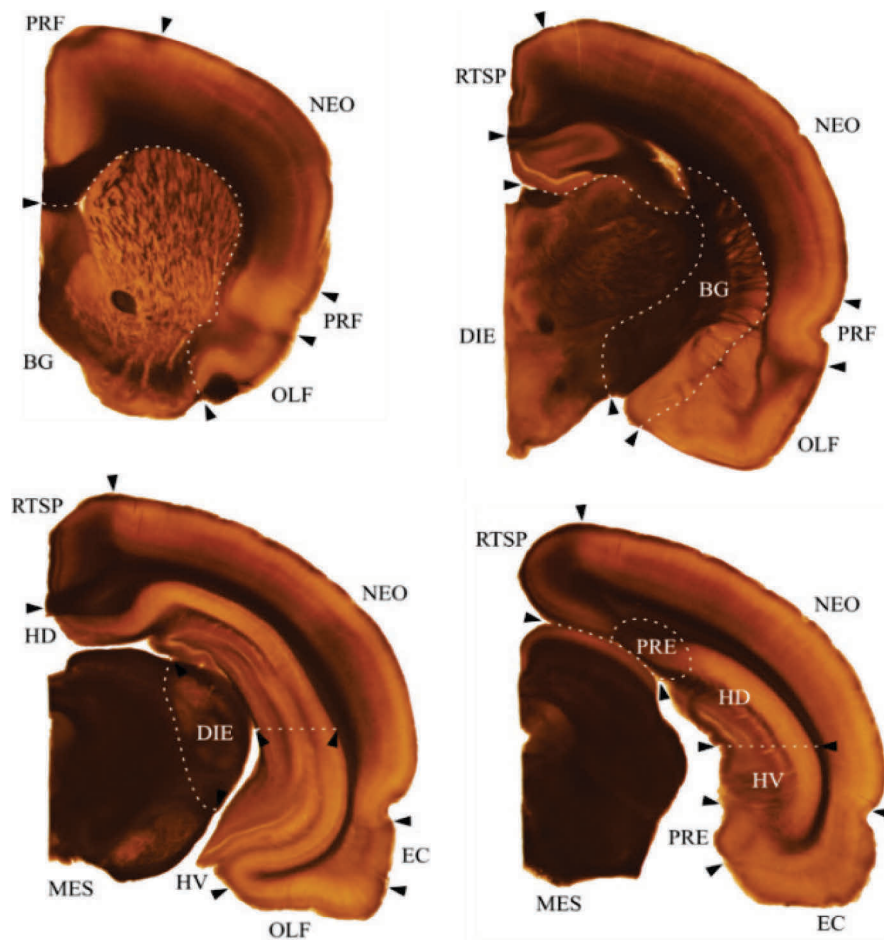


Figure 9. Schematic of dissection of representative sections. – There are approximately 50 x 200 μm sections of rat brain that are microdissected in the frontal plane. The sections are organized from rostral to caudal.

In Figure 9., I chose 4 representative pictures of frontal plane sections organized from rostral to caudal in order to demonstrate how the structures are divided using microknife.

4.1.3. Homogenization

Prior to homogenization, the weight of all samples was established using analytical weights. 14 structures were manually homogenized in diluted solution (DS). The homogenization process took under 5 minutes in most cases and under 40 min in all cases.

4.1.3.1. Transfer of homogenates

The homogenates were then transferred using plastic pipette to either 5 ml Eppendorf tubes or 50 ml falcon tubes according to their volume. 5% DAPI was then added to each of the samples to visualize cell nuclei.

4.1.4. Staining and counting

DAPI immediately binds to DNA so the sample is ready to be counted under fluorescent microscope using Neubauer counting chamber. DAPI emits blue light, UV filter is used to count cell nuclei. The homogenate needs to be thoroughly shaken before pipetting to Neubauer chamber because cell nuclei sediment very quickly. For every count, 10 µl aliquot is pipetted to each side of the Neubauer chamber. Every structure was counted at least 4 times and if the coefficient of variation (CV) is greater than 15 %, the sample is counted as many times as needed for the CV to be equal or under 15 %. The CV is counted using free software Statistics Calculator Pro. The mean cell count is determined from all of the counted aliquots and then multiplied by 10000*sample volume. This number then represents the total number of cells in one hemisphere of the respective structure.

4.1.4.1. NeuN staining

Approximately 1 million cells (50-200 µl depending on the sample) were put to a 500 µl Eppendorf tube and the rest of the Eppendorf tube was filled with PBS-TX.

Each of 14 samples was then thoroughly shaken and spined down in the centrifuge at 3600 rps for 2 min. In the next step, the supernatant was discarded and refilled with 450 μ l PBS-TX. The sample was thoroughly rinsed 3x in total because there were still some fragments of destroyed nuclei (<1 %) that would interfere with the sample quality. After rinsing, 450 μ l anti-NeuN antibody (1:1000) in PBS with 10% normal goat serum is added to every sample. All 14 samples are placed in the refrigerator for overnight incubation in a horizontal position.

The next day, all samples are again rinsed 3x in PBS-TX. After that, 450 μ l of PBS-TX with secondary antibody (anti-rabbit Alexa 594) is added and incubated in the refrigerator for 3-4 h. After incubation, each sample is washed again 3x and diluted to the required volume with 5% DAPI in PBS.

4.1.4.2. Determining neuronal cell counts and neuronal cell densities

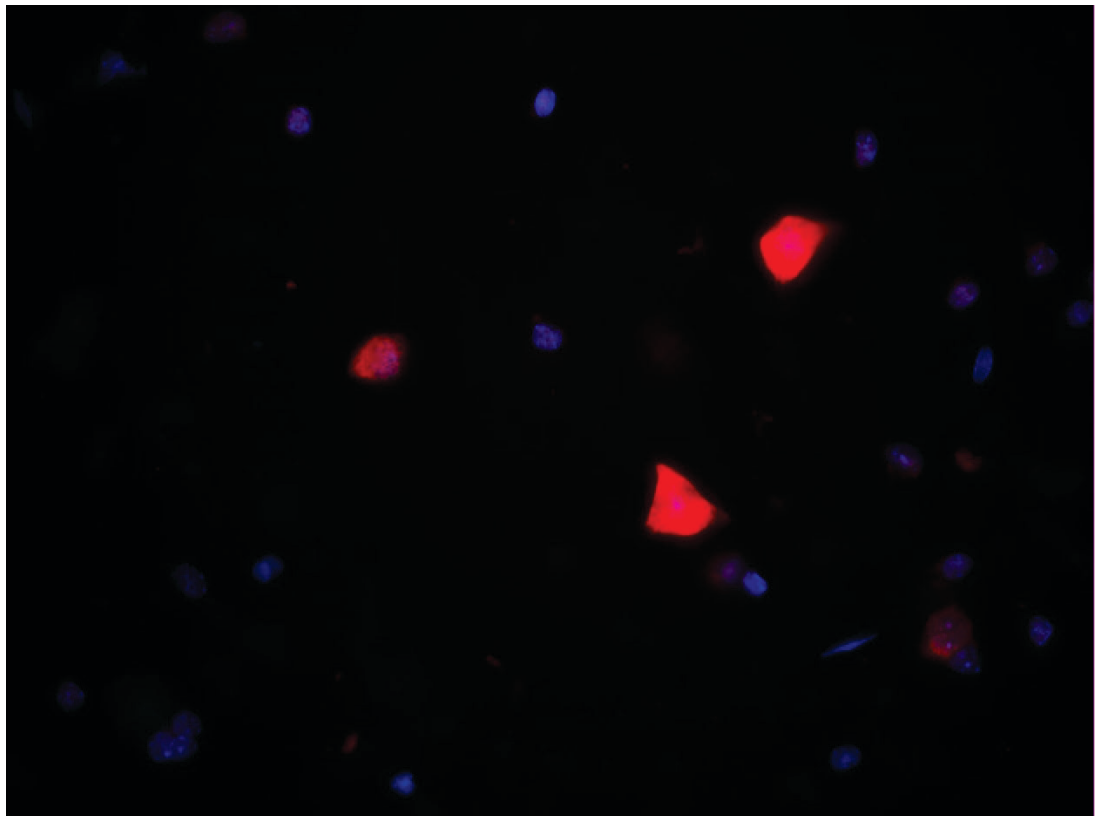


Figure 10. Example of NeuN staining in PRF of WT rats. (magnitude 40x) – Neurons are larger and stained with NeuN and emit red light, other cells are only stained with DAPI.

a. Sample preparation

As mentioned above, Eppendorf tubes were filled with 450 μ l of PBS-TX and contained nuclei suspension of respective structures stained with DAPI and NeuN. The solution is then pipetted up and down at least 5 times for the sample to be isotropic. 10 μ l of the sample was then pipetted to the Neubauer counting chamber and counted using fluorescent microscope as shown in the Figure 10.

b. Establishing total neuronal counts

To estimate the total number of neurons in a structure, 500 nuclei were counted and the ratio of neuronal (DAPI+, NeuN+) to non-neuronal cell nuclei (DAPI+, NeuN-) was determined. To estimate the total number of neurons and non-neuronal cells in a structure, neuronal and non-neuronal fraction was multiplied by total cell number, respectively.

4.2. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.03. Two-way ANOVA was used to compare all 14 structures in TgF-344 AD brains and wildtype controls. Data were tested for normal and log-normal distribution by the Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov test. Data that did not pass any of the normality tests but passed lognormality tests were log-transformed prior to statistical analysis.

4.2.1. IQR method

An outlier analysis was performed using the standard 1.5 interquartile change rule (IQR) method (1.5 times the interquartile range). The interquartile range is an indicator of how the data is spread about the median and is useful in determining outliers. Each dataset is represented by its five-number summary namely the minimum or lowest value of the dataset, the first quartile Q1 (a quarter of all data), the median of the data set, the third quartile Q3 (three-quarters of all data) and the maximum or highest value of the data set. IQR 1.5 is counted by subtracting the first quartile from the third quartile ($IQR = Q3 - Q1$). The number 1.5 affects the sensitivity of the range. 1.5 is a universally

accepted constant. If the scale was bigger, the outliers would be considered as data points. On the other hand, a smaller number would lead to wrongly assessing some data to outliers (Mishra et al., 2019).

4.2.2. Post-hoc test

Two-stage step-up method of Benjamini, Krieger and Yekutieli was performed to correct for multiple comparisons since I compared 14 structures of TgF-344 AD and wild type rat brain.

5. RESULTS

All numbers stated in results correspond to one hemisphere. Because of a low number of samples, I decided to not include OB in this analysis.

Table 2. Neuronal, non-neuronal numbers and total cell densities (CD) and their standard deviations (SD) in 19 months old TgF-344 AD rats.

	n	neurons	SD	non-neuronal cells	SD	CD (cells/g)	SD
PRF	7	1735656,143	321632	4963759,667	414927	1,1E+08	1,5E+07
OLF	7	1544404,333	449604	4386584,833	738857	1,2E+08	3,9E+07
EC	7	564733,3333	186779	1836674	639447	1,5E+08	5,7E+07
MES	7	1723476,857	354230	5674403,143	688543	1,1E+08	3,8E+07
DIE	7	1343699,333	326986	6555327,714	1165507	1,1E+08	9582709
HD	7	1109226	33010,2	3701930,667	228176	1,4E+08	3,2E+07
HV	7	468117,4429	141583	1968692,667	149317	8,7E+07	5,8E+07
PRE	7	302723,3286	79232,3	574860,4	203391	1,4E+08	9,6E+07
BG	7	3913067,857	586263	9982827,571	2546602	1,3E+08	6,1E+07
NEO	7	5465297,857	1639171	13602654,71	2097979	1E+08	5,1E+07
RTSP	7	601220,3571	92808,6	1561462	402680	1,1E+08	6,9E+07
CB	7	70940029,57	1,3E+07	34076524,57	1,4E+07	639164	119744
BSTEM	7	31638666,67	6163267	86518448	3,5E+07	1026961	169068

As illustrated in Table 2., there are 7 19 months old TgF-344 AD rats. The table represents mean number of neurons, non-neuronal cells and total cell density of each structure – OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM as well as their SD.

Table 3. Neuronal, non-neuronal numbers and total cell densities (CD) and their standard deviations (SD) in 19 months old WT rats.

	n	neurons	SD	non-neuronal cells	SD	CD (cells/g)	SD
PRF	8	2469237	407808	4653737,875	1141226	9,9E+07	1,9E+07
OLF	8	1548095,857	264313	3614213,25	667635	1E+08	1,9E+07
EC	8	663274,6429	365123	1452822,5	797362	1,3E+08	8E+07
MES	8	2087297,143	274125	5444902,5	938638	1E+08	1,4E+07
DIE	8	1851027,667	268592	5072118,5	592955	1E+08	8629485
HD	8	1551606,667	98449,9	3422311,25	419514	1,1E+08	1,7E+07
HV	8	710135,3571	95273,2	1622266,875	340379	9,2E+07	1,4E+07
PRE	6	288740,3167	62752,4	473915,1667	138103	1,4E+08	6,7E+07
BG	8	3785382,571	957480	8701500,375	2134797	9,5E+07	3,4E+07
NEO	8	5659257,143	835795	10699888,75	1559058	9,2E+07	1,7E+07
RTSP	8	851205,1429	254159	1419843,5	279974	1,1E+08	1,4E+07
CB	8	85971632,86	1,9E+07	54976088,63	4,2E+07	864063	193439
BSTEM	8	25028371,43	4981309	86510750	1E+07	1094685	449759

As illustrated in Table 3., there are 8 19 months old WT rats. The table represents mean number of neurons, non-neuronal cells and total cell density of each structure – OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM as well as their SD.

Table 4. Neuronal, non-neuronal numbers and total cell densities (CD) and their standard deviations (SD) in 6 months old TgF-344 AD rats.

	n	neurons	SD	non-neuronal cells	SD	CD	SD
PRF	5	1688107,2	529454	5031312	553285	8,5E+07	7530745
OLF	5	1436876,4	447797	3290206,5	339236	9,3E+07	1E+07
EC	5	567362,26	215704	1179311,25	434145	7,9E+07	3,1E+07
MES	5	2080148,2	1045018	4794814,5	665048	8,6E+07	1,5E+07
DIE	5	1362511,8	188971	4158118,25	277990	1,1E+08	2,7E+07
HD	5	985809,1	279276	2810486	411230	8,9E+07	2,8E+07
HV	5	523421,1	113533	1473008,75	296717	9,7E+07	8544176
PRE	5	287330,46	43579,4	395003,35	102905	1,4E+08	1,5E+07
BG	5	3823195,4	519443	9200200,25	1300180	9,1E+07	2,7E+07
NEO	5	6028742	1307231	11934678,75	1994938	1E+08	1,5E+07
RTSP	5	639876,82	126184	1254747	434136	1E+08	2,1E+07
CB	5	67315876	1,5E+07	19736680	8423354	548441	125378
BSTEM	5	14943885	2160305	98666300	1,9E+07	879373	136379

As illustrated in Table 4., there are 5 6 months old TgF-344 AD rats. The table represents mean number of neurons, non-neuronal cells and total cell density of each structure – OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM as well as their SD.

Table 5. Neuronal, non-neuronal numbers and total cell densities (CD) and their standard deviations (SD) in 6 months old WT rats.

	n	neurons	SD	non-neuronal cells	SD	CD	SD
PRF	4	2501437	351634	3656915,25	610354	1,1E+08	5354126
OLF	4	1587613,75	477289	3294511,25	430994	1,1E+08	1,6E+07
EC	4	766144,65	509865	1490105,25	704682	1,4E+08	6,1E+07
MES	4	1802242	279063	4548383	297989	1E+08	4732424
DIE	4	1610205,5	455743	4127294,75	724904	1,1E+08	1,3E+07
HD	4	1195403	195579	2493347	333574	1,1E+08	8212767
HV	4	775529,25	106768	1487283,25	333387	1,1E+08	1,3E+07
PRE	4	373781	109516	492694	150026	1,4E+08	2,9E+07
BG	4	3243249,75	570645	7253250,25	1109585	1,2E+08	1,8E+07
NEO	4	4193896,25	2660614	7845478,75	4496762	8E+07	4,5E+07
RTSP	4	770029,55	273243	1178345,5	383929	1,2E+08	2,6E+07
CB	4	66960874,75	5520800	17114423	2740669	606265	50940,9
BSTEM	4	5335289	7885848	23320023,5	2,6E+07	311261	409164

As illustrated in Table 5., there are 4 6 months old WT rats. The table represents mean number of neurons, non-neuronal cells and total cell density of each structure – OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM as well as their SD.

5.1. Neuronal numbers

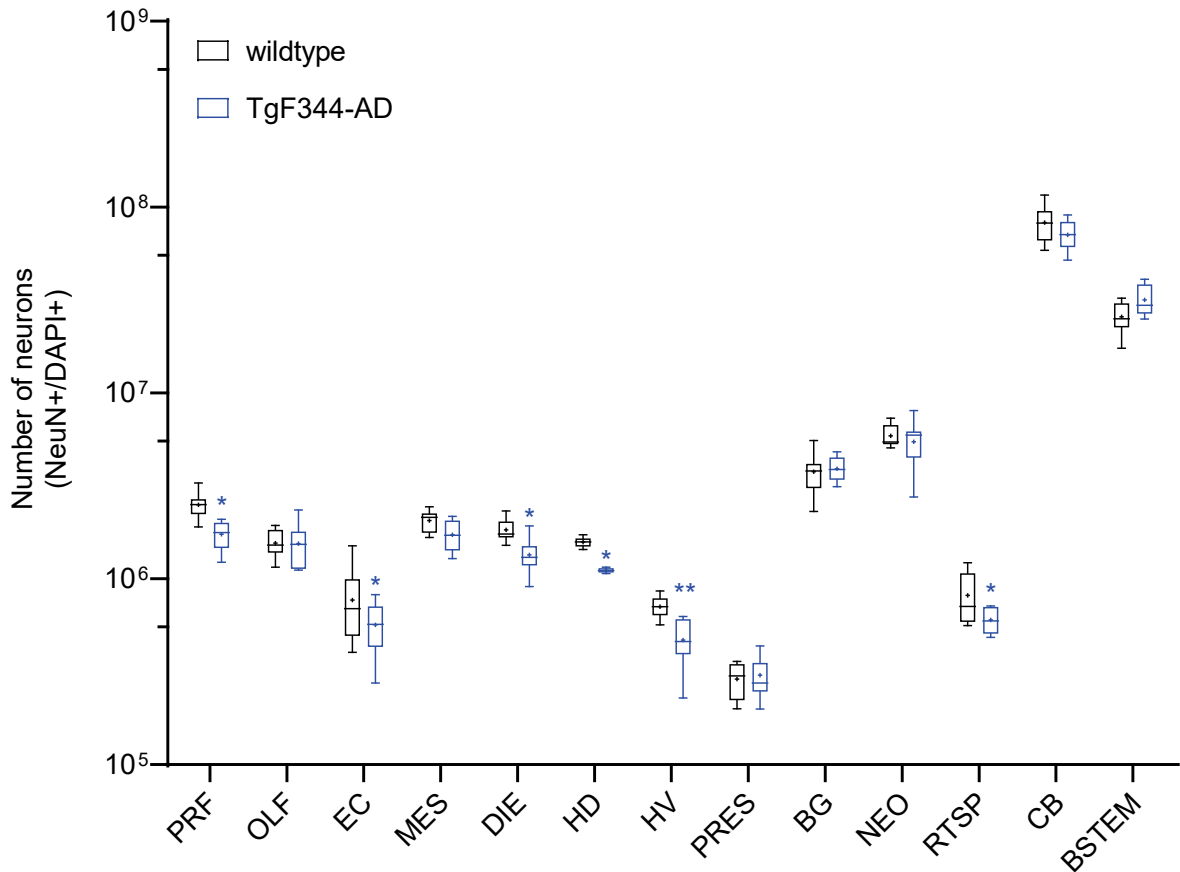


Figure 11. Neuronal numbers in 19 months old TgF-344 AD rats compared to wildtypes in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: *($p < 0.05$) **($p < 0.01$) *($p < 0.001$) **** ($p < 0.0001$). Neuronal loss is apparent in the TgF-344 AD compared to aged-matched wildtypes in the PRF, OLF, EC, HD, HV and RTSP. These structures are heavily affected in human AD brain as well.**

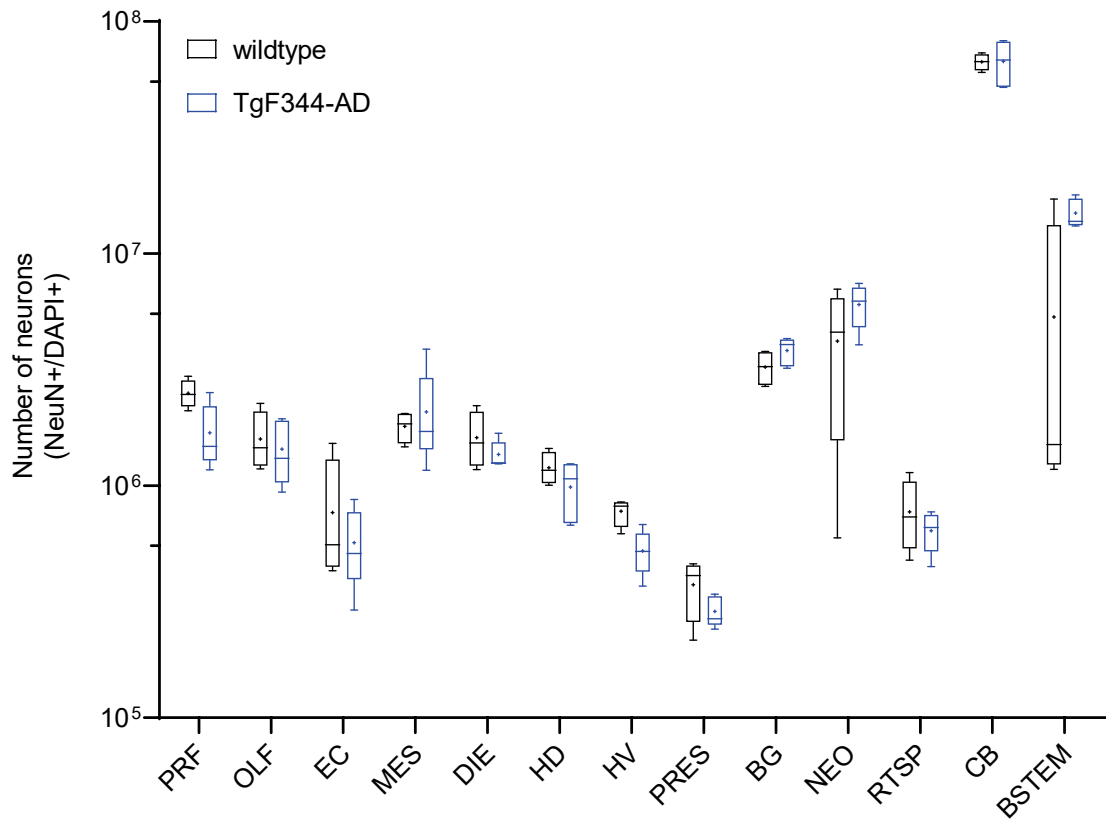


Figure 12. Neuronal numbers in 6 months old TgF-344 AD rats compared to wildtypes in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: *($p < 0,05$) **($p < 0,01$) *($p < 0,001$) **** ($p < 0,0001$). There are no significant changes in TgF-344 AD brains compared to WT rats even though the trend is that the numbers of neurons are slightly higher in the experimental group.**

5.2. Non-neuronal cell numbers

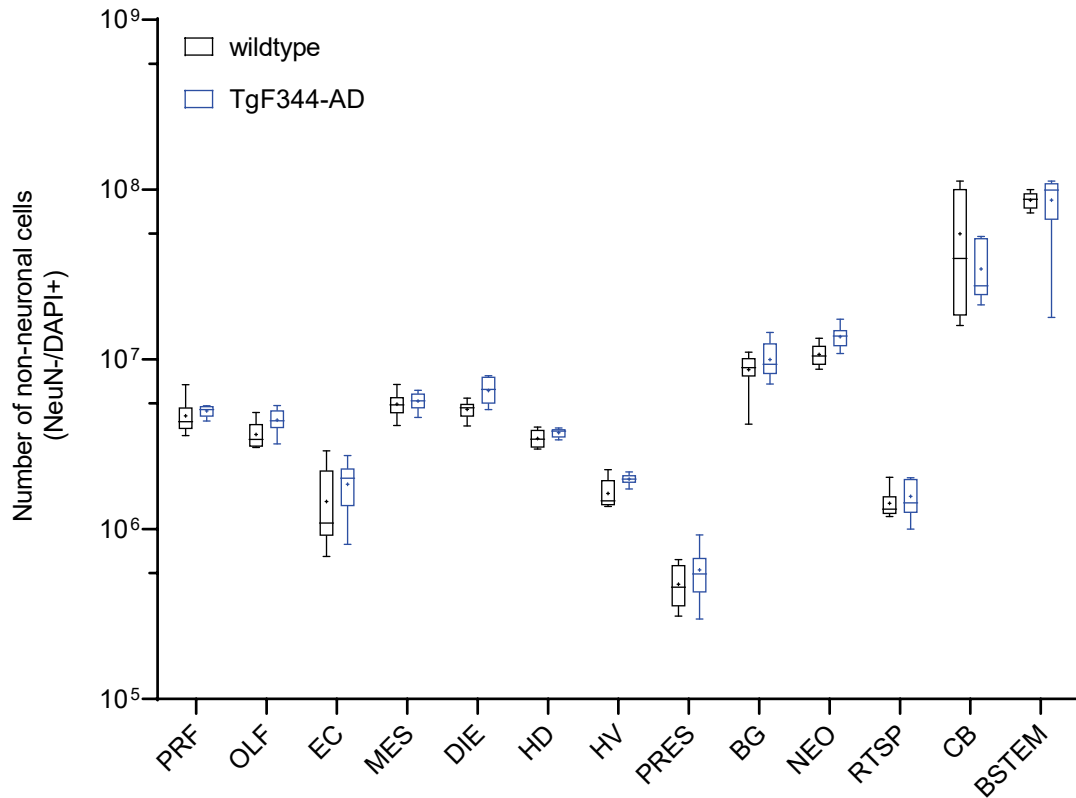


Figure 13. Non-neuronal numbers in 19 months old TgF-344 AD rats compared to wild-types in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: *($p < 0,05$) **($p < 0,01$) * ($p < 0,001$) **** ($p < 0,0001$). The data analysis did not reveal any significant changes in non-neuronal cells in TgF-344 AD rats compared to wild types even though the number of non-neuronal cells seems to be higher in most structures of TgF-344 AD brains.**

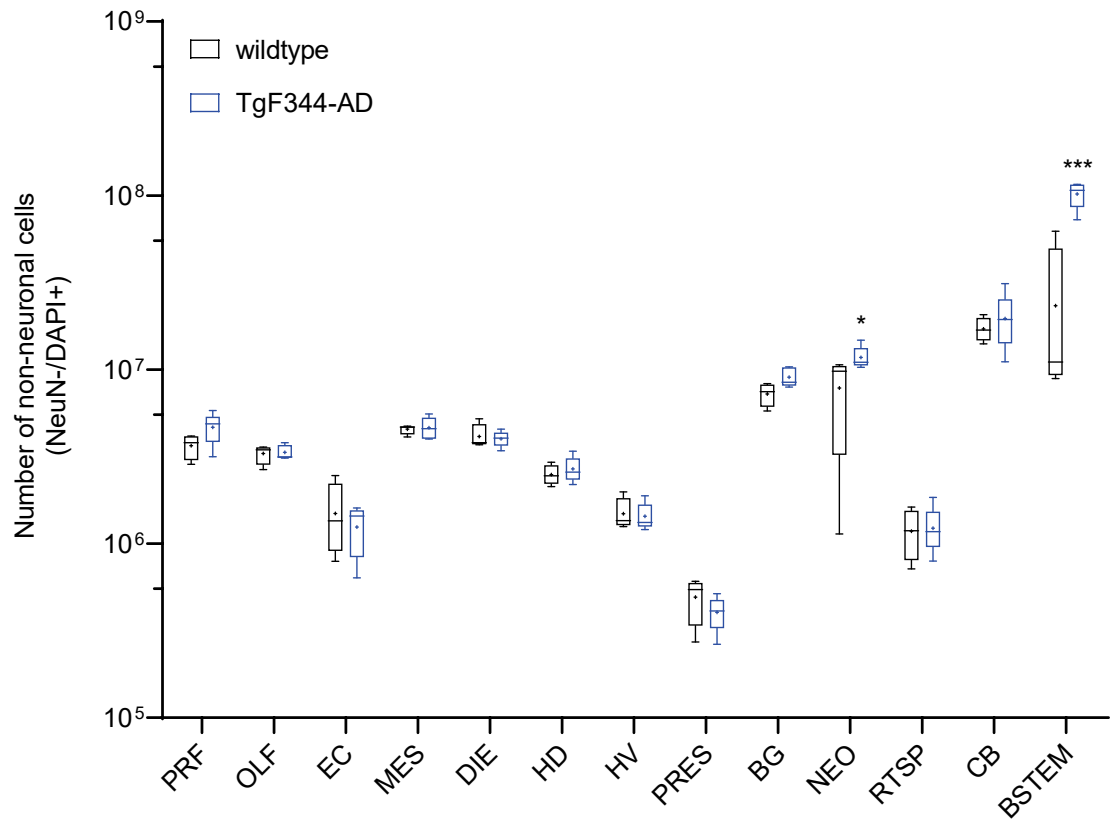


Figure 14. Non-neuronal numbers in 6 months old TgF-344 AD rats compared to wild-types in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: * (p<0,05) ** (p<0,01) * (p<0,001) **** (p<0,0001). There is elevated non-neuronal number in TgF-344 AD brains in NEO and BSTEM.**

5.3. Total cell density

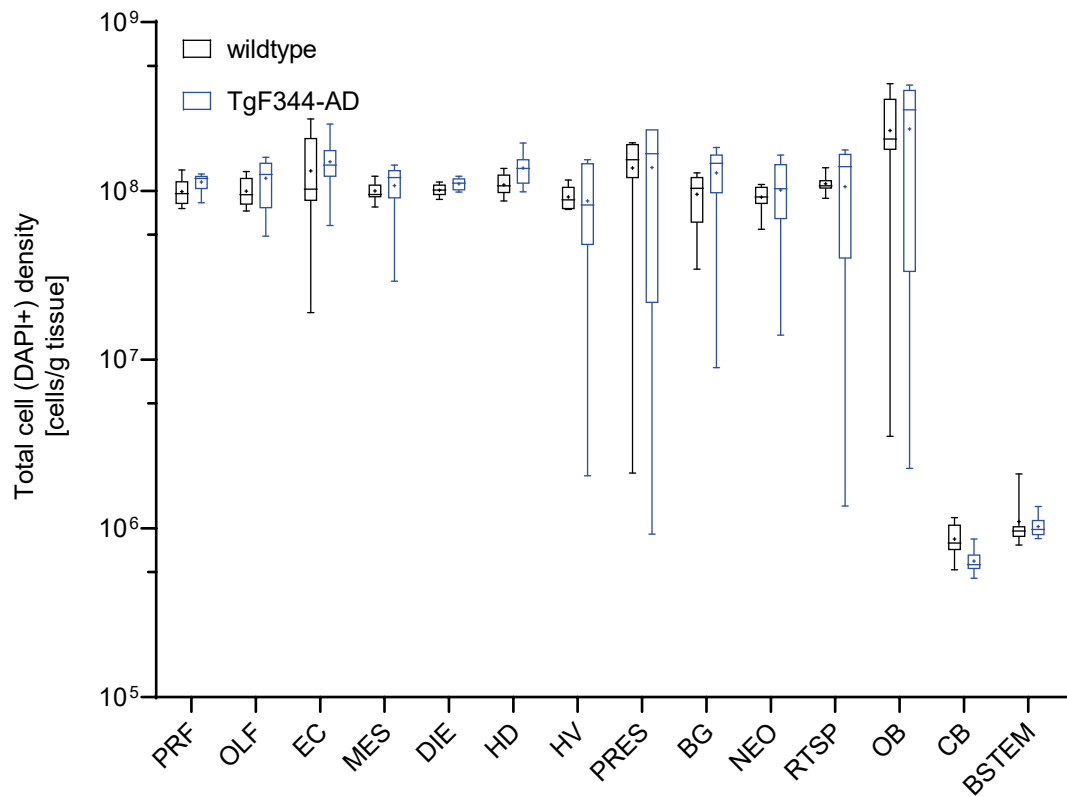


Figure 15. Cell densities in 19 months old TgF-344 AD rats compared to wild-types in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: *($p < 0,05$) **($p < 0,01$) *($p < 0,001$) **** ($p < 0,0001$). There is no significant difference in total cell density between TgF-344 AD and wild type rats. It appears that there is high interindividual variability within structures.**

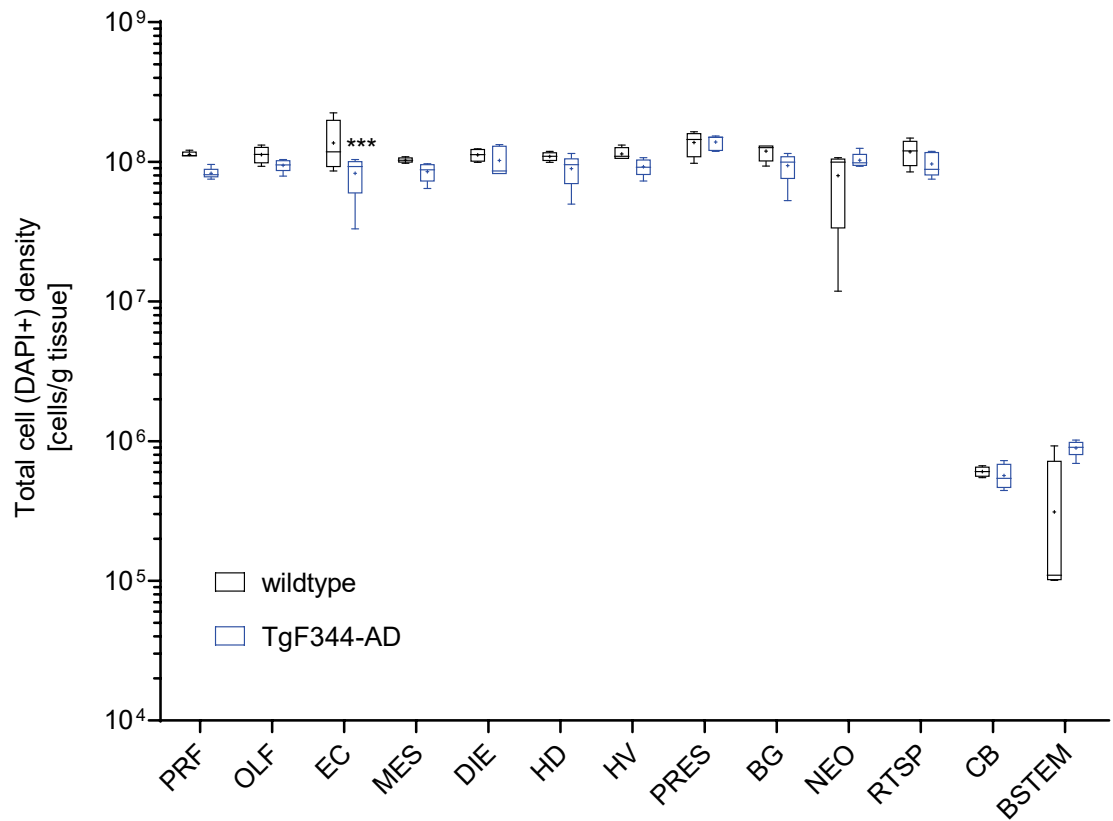


Figure 16. Cell densities in 6 months old TgF-344 AD rats compared to wild-types in 13 structures: OLF, PRF, BG, DIE, MES, HD, HV, EC, PRE, RTSP, NEO, CB and BSTEM. The levels of significance are marked: *($p < 0,05$) **($p < 0,01$) *($p < 0,001$) ****($p < 0,0001$). The seems to be significantly lower total cell density in TgF-344 AD brains.**

6. DISCUSSION

There is still a huge gap in understanding of cellular interactions underlying AD progress. Neuronal loss is a result of neurodegeneration and diminishes functional connectivity and therefore reflects on cognitive malfunction.

Before focusing on concrete data on neuronal loss, it is important to discuss the significance of neuronal loss in AD compared to synaptic loss. Studies have shown neuronal synaptic loss to occur early on during the MCI stage (Scheff et al., 2006). There is also apparent neuronal loss even in the MCI stage but the plasticity of the brain is able to make up for it, at least to a certain degree. (Barrio et al., 2008) In comparison to neuronal loss, synaptic loss seems to be the strongest correlate of cognitive decline. Scientists have focused on pathological features present in the early stages of AD in the past years to pinpoint an initial trigger of dementia. Importantly, synaptic loss does not necessarily mean neuronal death because neuronal nuclei can still survive if provided by neurotrophic factors. This means that quantifying synaptic loss does not equal quantifying neuronal loss. From another point of view, since the neurogenesis in humans does not occur or is extremely limited, neuronal loss could be the point in AD pathology that is irreversible whilst perhaps synaptic loss could still be managed.

There are multiple pathological events that contribute to reduced neuronal number e.g. A β , NFTs and chronic inflammation as discussed in the literary overview but we still lack knowledge about initial triggers that ultimately cause neuronal loss. The line between causality and correlation becomes complicated and blurry, because pathological hallmarks such as NFTs and SPs contribute to neuronal loss but neuronal loss exceeds them (Kril et al., 2002 ; Gómez-Isla et al., 1997). To finally determine the initial and main trigger of neuronal loss, it will take many more years of various experiments and it would be a useful start to provide quantitative data about selective structural vulnerability in AD.

6.1. Changes in neuronal and cell numbers and densities

6.1.1. Neuronal numbers

In my master's thesis, I shed more light to the quantitative cell analysis of a large number of structures to provide more information on the degree of neurodegeneration in each of the structures as opposed to the studies that only centred around a limited number of structures, usually neocortex (Gómez-Isla et al., 1997) or hippocampus (Padurariu et al., 2012). It is important to quantify neuronal loss in the affected structures as well as the ones who do not suffer a significant neuronal loss to be certain that the neurodegeneration of AD dementia is selective at least to a certain degree.

According to Figure 11., neuronal numbers were reduced in 19 months old TgF-344 AD brains in structures susceptible to AD early on – namely EC and HD which confirms what previous studies have already reported (Zallo et al., 2018 ; Kordower et al., 2001). Significant loss was also observed in PFC, one of the later damaged structures in AD. The present studies quite often focus on quantifying larger parts of neocortex and we need more data on specific parts such as PFC. Especially since neurodegenerative changes in PFC in AD has have been established many times (DeKosky & Scheff, 1990 ; Kashani et al., 2008). I also observed significant neuronal loss in RTSP. RTSP is connected to the hippocampal formation and plays role in learning and spatial navigation. Pathological changes in RTSP begin during the MCI stage (Nestor et al., 2003) but we lack data on neuronal loss in this structure. This is one of the main contribution of my data – because I examined a large number of structures, I can provide data on neuronal loss in less studied structures which nonetheless are important for AD pathology.

According to Figure 12., there are no significant changes between 6 months old TgF-344 AD and WT brains even though the experimental group seems to have elevated neuronal numbers. It is possible that AD-like pathology did not fully develop at 6 months yet and the neurons are not severely affected.

In my master's thesis, I also focused on structures that I did not expect a significant change in neuronal numbers in order to be certain that the selective vulnerability occurs in AD. Other studies reported that CB could have some protective mechanisms against AD (Xu et al., 2019) and neuronal numbers do not

seem to decrease in AD (Andersen & Andersen & Pakkenberg, 2012) which is in concordance with my data.

6.1.2. Non-neuronal cell numbers

As Figure 13. suggests, I did not observe any significant changes non-neuronal cell numbers even though non-neuronal numbers seem to be slightly elevated in 19 months old TgF-344 AD rats. In AD, chronic inflammation is one of the prominent AD pathology signs and leads to elevated numbers of microglia and astrocytes. It is possible that elevated numbers of certain glial subtypes could explain why non-neuronal counts in this experiment are slightly higher in TgF-344 AD brains compared to wild-types even though the result is not significant for any structure. More studies are needed to determine how glial numbers change during the progress of dementia due to AD. Interestingly, in Figure 14., the non-neuronal numbers are significantly elevated in BSTEM and NEO in the 6 months old experimental group.

6.1.3. Total cell density

In Figure 15., I did not report any significant changes in cell densities even though cell density seem to be slightly higher in 19 months old TgF-344 AD brains. This can be surprising because the shrinkage of AD brain could potentially lead to a higher cell density of AD brain. Also, in humans, some studies reported opposite trend, in the hippocampus, at least neuronal density is lower in AD compared to healthy controls (Padurariu et al., 2012 ; Barrio et al., 2008). On the other hand, in Figure 16., in EC, cell density is higher in 6 months old WT brain. EC is one of the first damaged structures in AD which could explain why it might shrink early on.

6.2. TgF-344 AD model

The TgF-344 AD model used in my master's project is based on overexpression of mutated human APP and PSEN-1 leading to a higher accumulation of A β and formation of SPs. According to my results, there seems to be a selective vulnerability in the same structures in TgF-344 AD and humans, the neuronal loss is significant in HD and HV, PRF. This could mean that the selective vulnerability is not dependent solely

on A β overexpression since there are other major pathological factors that play role in human AD and still the same structures are affected compared to TgF-344 AD.

We chose the TgF-344 AD model because it has been previously shown that this transgenic strain exhibits prominent AD pathologies such as A β and tau dysregulation, behavioural impairment and importantly, frank neuronal loss (Cohen et al., 2013).

6.3. Future directions in quantifying cell loss

There are multiple possible future directions in quantifying cell loss that could provide useful information about AD dementia and TgF-344 AD rat model. First, my master's project is a part of a bigger project that is focused on quantifying neurons in three different timepoints – 6 months, 12 months and 19 months. By quantifying different timepoints, we will correlate neuronal loss with the dementia progression and reveal which structures susceptible to AD struggle with neuronal loss first.

For a more detailed perspective, quantifying loss of individual neuronal subtypes seems a viable next step. In introduction, I summarized experiments on loss of parvalbumin neurons in basal forebrain. Disrupted Ca²⁺ levels are a major source of excitotoxicity in AD and loss of parvalbumin neurons should be examined in other structures as well. Also, in App^{NL-G-F} mice, it has been shown that stabilizing ryanodine receptor (RyR2) by enhancing calmodulin binding prevents neuronal loss (Nakamura et al., 2021). There are possible clinical implications of this finding since it provides a mechanism by which AD induced Ca²⁺ excitotoxicity could be less expanded. Calmodulin is a prominent Ca²⁺-binding protein in the brain just like parvalbumin. It would be interesting to quantify how calmodulin levels change in AD.

Another interesting aspect is intersex differences in AD susceptibility (Yao et al., 2009). There is a wide scope of theories from social to hormonal that aim to explain why women are more likely to develop AD than men. In my master's thesis, I only quantified neuronal loss in male TgF-344 AD rats, it would be interesting to compare the results with females as well.

So far, neurons received more attention than glial cells which should be explored more in the future. There are reports on glial malfunction in AD, e.g. involvement of microglia and astrocytes in chronic inflammation implying that their numbers could be elevated in AD. According to my results, the nonneuronal numbers in TgF-344 AD are not significantly elevated but their numbers tend to be higher compared to the control group. Also, in recent years, oligodendrocytes have been implicated in AD pathology due to myelin dysregulation (McKenzie et al., 2017). In AD, glycolytic genes that regulate glucose metabolism are most severely impaired in oligodendrocytes compared to other glial subtypes and even neurons (Saito et al., 2021).

6.4. Limitations

There are several limitation of these data and the methodology that was chosen. First, with IF, we can only provide data on cell and neuronal loss and density but since the whole structure is homogenized, it is impossible to obtain any morphological data compared to stereological techniques that are more time-demanding. It would be useful to obtain data on how the number of dendrites changes during the progression of AD or perhaps observe the shrinkage of neuronal soma.

In my master's project, I only focused on quantifying cell changes in male rat brains and it would be interesting to see whether the changes are more prominent in female rat brains.

7. CONCLUSION

The goal of this master's thesis was to establish changes in neuronal and nonneuronal counts in TgF-344 AD rat brains as well as total cell density of TgF-344 AD and wild type rat brains. In total, 24 rat brains and 13 structures of 6 and 19 months old TgF-344 AD (n=5,7) brains and matched wildtype controls (n=4,8) were quantified.

These data show a significant neuronal loss in 19 months old TgF-344 AD in structures that has been reported to be susceptible to AD in AD patients early on – EC, HD, HV and in later stages – PRF. I also reported neuronal loss in RTSP that has not been previously quantified in TgF-344 AD rats.

I did not observe any significant changes in non-neuronal cell numbers in 19 months old TgF-344 AD. It is possible that diminished numbers of microglia or astrocytes could explain why non-neuronal counts in this experiment are slightly higher in TgF-344 AD brains compared to wild-types. At least according to these data, the result is not significant. More studies are needed to establish how glial numbers change during AD. On the other hand, in 6 months old TgF-344 AD brain non-neuronal cells were significantly higher in BSTEM and NEO.

Regarding the cell densities, I did not observe any significant changes 19 months old TgF-344 AD. There seems to be high interindividual variability in our samples so that could have attributed to an insignificant result. In 6 months old TgF-344 AD, total cell density is lower in the experimental brains in EC which corresponds with possible brain tissue shrinkage.

Perhaps even more important than a quantification of neuronal loss in AD, these results contribute to understanding selective vulnerability of certain structures in AD. One of the possible implications of these results could be that since the TgF-344 AD model used in the experiment is primarily based on A β pathology and the affected structures correspond to human AD brains, the selective vulnerability appears regardless whether the model is solely based on A β pathology or on other pathologies as well.

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secondary source is marked with *

All images and photos are either created by me or our lab.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Ach	acetylcholine
ACH	amyloid cascade hypothesis
AChE	acetylcholinesterase
APP	amyloid precursor protein
A β	beta amyloid
BF	basal forebrain
BG	basal ganglia
BSTEM	brainstem
CB	cerebellum
DIE	diencephalon
DMN	default mode network
e.g.	for example (exempli gratia)
EC	entorhinal cortex
FAD	familial AD
FDA	Food and Drug Administration
GLUT4	glucose transporters 4
GSK-3	glycogen synthase kinase 3
GWAS	genome wide association study
HD	dorsal hippocampus
HV	ventral hippocampus
CHT	high-affinity choline transporter
i.e.	that is
IF	isotropic fractionator
IQR	interquartile range
mAChRs	muscarinic acetylcholine receptors
MAPs	microtubule associated proteins
MCI	mild cognitive impairment
MES	mesencephalon
nAChRs	nicotinic acetylcholine receptors

NEO	rest of cortex
NFTs	neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
OB	olfactory bulbs
OLF	olfactory cortex
PFA	paraformaldehyde
PLA ₂	phospholipase A ₂
PRE	presubiculum
PRF	prefrontal cortex
PSEN 1	presenilin-1
PSEN 2	presenilin-2
PVS	perivascular spaces
ROS	reactive oxygen species
RTSP	retrosplenial cortex
RyR2	ryanodine receptor
SAD	sporadic AD
SPs	amyloid (senile) plaques
STZ	streptozotocin
VaD	vascular dementia
VGluT	vesicular glutamate transporter