Analysis of tau protein spread in rat brain as a model of Alzheimer's disease

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Analysis of tau protein spread in rat brain as a model of Alzheimer's disease

Graduation thesis

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This Master's thesis was created at the Croatian Institute for Brain Research, Laboratory for developmental neuropathology under the supervision of Professor Goran Šimić, MD, PhD.

The Master's thesis is submitted to the Department of Biology, Faculty of Science, University of Zagreb for the purpose of attaining the academic degree Master of Molecular Biology.

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Analiza širenja proteina tau u mozgu štakora kao modela Alzheimerove bolesti

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Alzheimerova bolest (AB) je neurodegenerativna bolest i najčešći uzrok sindroma demencije. Postoje mnoge teorije o mogućim mehanizmima nastanka i širenja AB-a, od kojih je jedna tau hipoteza. Ta teorija pretpostavlja da hiperfosforilacija tau proteina dovodi do njihovog pogrešnog smatanja, oligomerizacije i nakupljanja, što remeti strukturu i funkciju mikrotubula te u konačnici odumiranja sinapsi i neurona. U ovom radu smo istražili promjene i širenje tau fibrila i tau oligomera inokuliranih u medijalni dio entorinalne moždane kore devet Wistar štakora. Za imunohistokemijsku vizualizaciju fosforiliranog tau proteina na epitopu Ser202/Thr205 korišteno je AT8 protutijelo. Tri koronalne razine (udaljenost od bregme -8,04, -6,84 i -5,64 mm) analizirane su 4, 8 i 11 mjeseci nakon inokulacije. Glavni nalaz je bio taj da su u životinjama kojima su inokulirane tau fibrile uočene značajne neurofibrilarne promjene i širenje tau fibrila, dok se takve promjene nisu dogodile kod životinja kojima su inokulirani tau oligomeri ili otopina fosfatnog pufera (kontrole). Navedeni rezultati upućuju da bi tau fibrile mogle biti uzročnik nastanka AB-a, pa stoga zaslužuju daljnju validaciju i detaljniju analizu u budućim istraživanjima.

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University of Zagreb
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Analysis of tau protein spread in the brain of a rat model of Alzheimer's disease

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Alzheimer's disease (AD) is a neurodegenerative disease and the most common primary cause of dementia syndrome. Many theories exist regarding possible mechanisms of development and progression of AD, one of which is the tau hypothesis. This theory suggests that the hyperphosphorylated tau proteins misfold, oligomerize and aggregate, disrupting microtubule structure and function ultimately leading to the death of synapses and neurons. In this work we investigated changes and spreading of tau fibrils and tau oligomers that were inoculated into the medial entorhinal cortex of nine Wistar rats. The phosphorylated tau proteins were visualised by using the AT8 antibody, which binds to Ser202/Thr205 epitope of tau. Three coronal levels (-8.04, -6.84, and -5.64 mm from bregma) were analysed at 4, 8, and 11-month post-inoculation. The main finding was that the animals inoculated with the tau fibrils displayed significantly greater amount of neurofibrillary changes and spread of tau fibrils, while such changes did not occur in animals inoculated with tau oligomers and phosphate buffer saline (controls). These findings suggest that tau fibrils may be the true culprit in AD and thus deserve further validation and a more detailed analysis in future studies.

(41 pages, 15 figures, 2 tables, 54 references, original in: English)

Thesis deposited in the Central Biological Library

Key words: Alzheimer’s Disease, Dementia, Immunohistochemistry, Tau Protein

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List of Abbreviations

Aβ	amyloid-β
AD	Alzheimer's disease
ADRDA	Alzheimer’s Disease and Related Disorders Association
APOE	apolipoprotein E
APP	amyloid precursor protein
BACE1	β-secretases
BSA	bovine serum albumin
ChAT	choline acetyltransferase
CSF	cerebrospinal fluid
CTF	C-terminal fragment
CTR	control
DAB	3,3′-Diaminobenzidine
DNA	deoxyribonucleic acid
EC	entorhinal cortex
GABA	gamma-aminobutyric acid
HC	hippocampus
MAP	microtubule-associated proteins
MAPT	microtubule associated protein tau
MCI	mild cognitive impairment
MMSE	Mini Mental State Examination
MRI	magnetic resonance imaging
MTB	microtubule-binding
NIA-AA	National Institute on Aging - Alzheimer’s Association
NFT	neurofibrillary tangles
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMDA	*N*-Methyl-d-aspartate
NT	neuropil threads

O-GlcNAc	O-linked N-acetylglucosamine
PBS	phosphate-buffered saline
PDPK	proline-directed protein kinases
PET	positron-emission tomography
PHF	paired helical filaments
PrP^{C}	normal prion protein
PrP^{Sc}	infectious isoform of PrP
PSEN1	presenilin 1
PSEN2	presenilin 2
ROS	reactive oxygen species
SF	straight filaments
SPECT	single-photon emission computed tomography
TF	tau fibrils
TO	tau oligomers
TPK	tyrosine protein kinases
WHO	World Health Organization

Contents

1 Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) was first described in 1906 by Alois Alzheimer, who based his description on the case of a 51-years old patient Auguste Deter (Soria Lopez *et al*., 2019). Alzheimer's is the most common cause of dementia, causing up to 75% of dementia cases, and according to World Health Organization (WHO), it is a global health priority (Lane *et al*., 2018).

AD is a progressive neurodegenerative disorder characterized by the intracellular accumulation of neurofibrillary tangles (NFT) consisting of hyperphosphorylated tau proteins and extracellular accumulation of amyloid-β (Aβ) plaques (Bertram and Tanzi, 2008). Furthermore, loss of synapses, neurons, and white matter is also related to AD (Reitz and Mayeux, 2016), which is illustrated in Figure 1. AD is regarded as a multifactorial disease, with several risk factors, with the major one being age of a person (Korolev, 2014). Clinically, symptoms of AD can be roughly divided into three groups: the first group includes memory loss, loss of executive functions, and difficulties with language, the second group includes various psychiatric and behavioural issues, and the third group includes symptoms that are mainly focused on problems with living day to day (Burns and Iliffe, 2009).

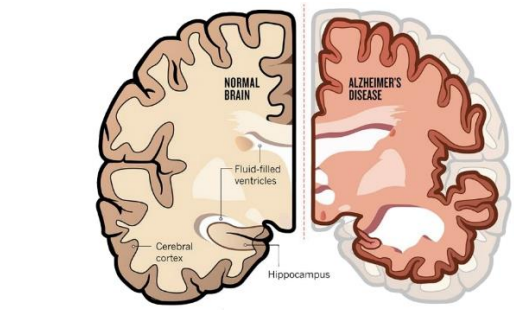

Figure 1. Differences in gross anatomy between a normal brain and the brain of Alzheimer's patient (https://www.flickr.com/photos/rik-williams)

For clinical diagnosis of AD, criteria revised by the National Institute on Aging - Alzheimer's Association (NIA-AA) workgroup are used; the criteria were previously established by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) workgroup back in 1984. The revised criteria differ between cognitive impairment caused by AD and impairment caused by other dementias and events such as head trauma. The guidelines also implemented use of biomarkers in the diagnosis of the AD (Albert *et al.*, 2011, Jack *et al.*, 2011).

To clinically diagnose AD, Mini Mental State Examination (MMSE) is one of the most common tests for detection of AD in patients who suffer from the mild cognitive impairment (MCI), together with a neurological assessment (Arevalo-Rodriguez *et al.*, 2015). Other diagnostics include magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron-emission tomography (PET) imaging (Figure 2.). All three of those in fact measure some kind of biomarker related to AD; MRI and SPECT measure neuronal injury whereas PET measures the evidence of Aβ deposition. Aβ depositions, as well as tau depositions, can also be measured in the cerebrospinal fluid (CSF) (Albert *et al.*, 2011).

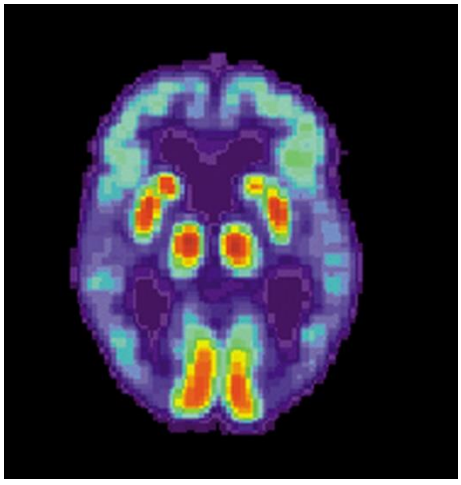

Figure 2. PET scan of a brain affected by Alzheimer's disease showing loss of temporal lobe function (https://en.wikipedia.org/wiki/File:PET_Alzheimer.jpg)

Biomarkers are used in order to establish the etiology of the symptoms, possible progression of the dementia, and to assume the certainty that the cause of the dementia is precisely AD. However, more biomarker studies, as well as the standardization of the biomarkers are needed for better diagnostics and hopefully better therapeutic approach (Jack *et al.*, 2011).

1.1.1 Pathogenesis of Alzheimer's disease

Main characteristics of AD are amyloid plaques and NFT, followed by activation of microglia, while neuropil threads, dystrophic neurites, and associated astrogliosis could also be present. These pathological changes lead to the loss of synaptic and neuronal activity, which further lead to macroscopic atrophy (Lane *et al*., 2018). Plaques, NFT, and loss of neurons and other brain cells can be present in many regions of the brain, including neocortex, hippocampus, and amygdala in a greater extent and thalamic medial nucleus, dorsal tegmentum, and locus coeruleus in a lesser extent. Other brain regions can also be affected (Wenk, 2003). On the other hand, it is possible for a person without dementia to also develop NFT-s; some cognitive difficulties are, however, noted in these patients. The main difference in brains of these particular patients was the absence of paired helical filaments (PHF) and a lower amount of amyloid plaques (Šimić *et al*., 1988).

Microscopically, amyloid plaques (formerly known as senile plaques) accumulate in the extracellular fluid, while NFT accumulate intracellularly, as illustrated in Figure 3. (Pavlović *et al*., 2007). Amyloid plaques form an oval- shape in the brain and during histological analysis, these plaques bind to the colour Congo red (Gandy, 2005).

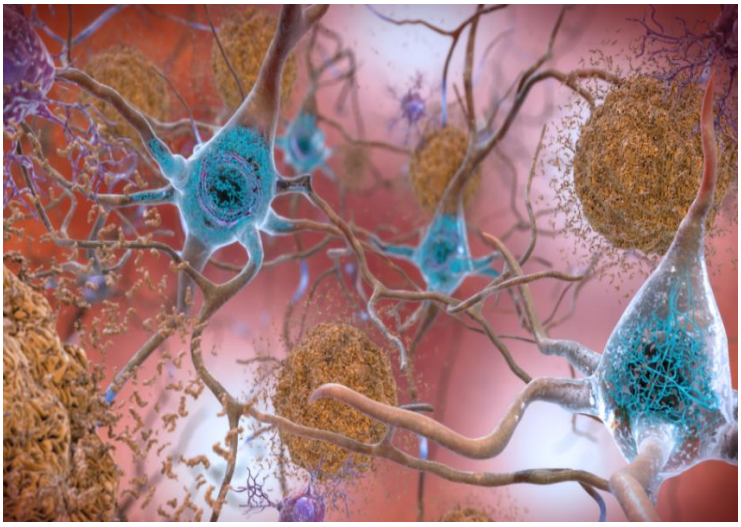

Figure 3. A depiction of extracellular amyloid plaques (shown in brown) and intracellular NFTs (shown in blue) in the brain affected by Alzheimer's disease (https://www.flickr.com/photos/nihgov/)

Electron microscopy shows that tangles consist of PHFs, which are comprised of tau proteins. For the identification of these proteins, antibodies that bind to specific epitopes are used (Selkoe, 2001), some of which are showed in Figure 4.

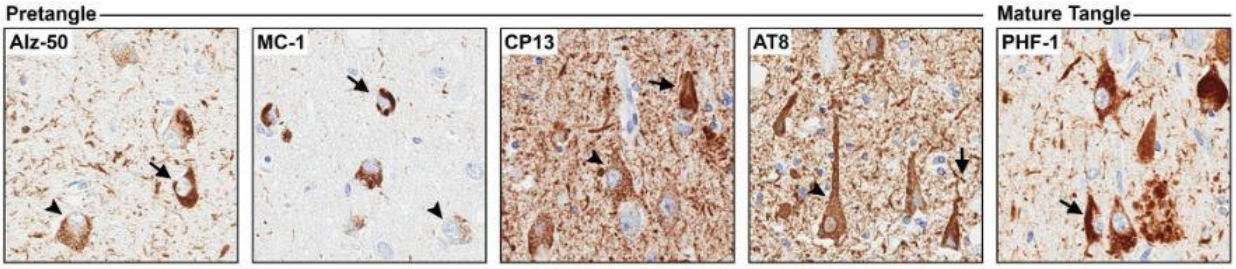

Figure 4. NFT visualised using different antibodies (Alz-50, MC-1, CP13, AT8, PHF-1) and staining techniques. All samples come from a hippocampus of AD brains. Scale = 25μm; from Moloney *et al.*, 2021, under Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) license.

In the cerebral cortex and hippocampus, the reduction of choline acetyltransferase (ChAT) activity has often been noted in the cases of AD (Wenk, 2003). Furthermore, basal nucleus of Meynert exhibits a neuronal loss; this particular nucleus is also involved in the cholinergic projection pathway (together with septal nuclei), the main pathway that provides cholinergic input to cerebral cortex. As damaged cortical cells fail to deliver neurotrophic factors to their respective low- and high-affinity receptors in terminals of the basal forebrain neurons, the expression of *CHAT* gene in the basal nucleus neuronsdecreases (Wenk, 2003).

Other neurotransmitter systems are also affected during the course of AD and so lower levels of serotonin, noradrenalin, gamma-aminobutyric acid (GABA), glutamate, substance P, and neuropeptide Y have been reported (Pavlović *et al.*, 2007).

1.1.2 Epidemiology of Alzheimer's disease

According to a 2018 paper, there are currently 44 million patients suffering from the AD in the world, and this number is predicted to triple by the end of 2050, which would mean that, in only 30 years, a total number of patients would be intimidating 132 million. The largest increase, it is theorized, would be in mid- and low-income countries (Lane *et al*., 2018). An older study set out to investigate differences in the incidence of AD between men and women; it showed that after the age of 70, women had a much higher possibility of developing AD (Andersen *et al*., 1999).

The biggest risk factors for AD are cardiovascular diseases, hypertension, smoking, type II diabetes, obesity, and traumatic head injuries, while education, physical activity, and Mediterranean diet are regarded as protective factors (Mayeux and Stern, 2012).

AD is a leading cause of dementias globally and as such a tremendous burden on healthcare systems, causing billions of dollars in treatment (Reitz *et al*., 2011).

It is known that AD causes shorter life expectancy, however these predictions also depend on the age of the patient. If they are diagnosed in their 60s, predicted life span is up to ten years, while this span is dramatically reduced to only three years for patients diagnosed at age 90 or higher (Zanetti *et al*., 2009).

1.2 Causes of Alzheimer's disease

The primary cause of AD is not known. However, there are many theories about the possible causes, such as theories based on aging-related phenomena, degenerative changes, genetics, and environmental causes (Armstrong, 2013). Most prominent theories include amyloid hypothesis and tau hypothesis; the amyloid theory suggests that Aβ protein aggregates and forms plaques that disrupt normal functions of the brain, while tau theory suggests the cause of tangle formation is hyperphosphorylation of tau proteins (Tiwari *et al.*, 2019).

1.2.1 Genetic causes

Over 90% of AD cases are sporadic, and not genetic, however, understanding the genetics could help us in the overall understanding of the disease (Bekris *et al.*, 2010). In early-onset AD (patients younger than 65 years of age), there are three main genes responsible for the onset of AD: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) gene. Another gene, apolipoprotein E (*APOE*) E4 allele, is responsible for the late-onset AD (patients older than 65 years of age) (Bagyinszky *et al.*, 2014). These four genes are responsible for up to 50% of AD inheritability (Tanzi, 2012).

APP, *PSEN1*, and *PSEN2* all lead to alterations in the production of Aβ protein, as well as *APOE* (Bagyinszky *et al.*, 2014).

Possible epigenetic causes have also been investigated in AD; DNA methylation and histone modifications were noted as possibilities. For example, DNA hypermethylation of the *HERT* gene was described in a histological study of AD patients, whereas gene coding for neprilysin (protein that has a role in the degradation of Aβ) was found to be affected by both methylation and histone modification (Alagiakrishnan *et al.*, 2012). A comprehensive review of epigenetic mechanisms in AD also indicated to other histone modifications, most notably, hyperphosphorylation of histone H3 (which plays a role in the activation of the mitotic machinery) in hippocampal neurons of AD patients (Mastroeni *et al.*, 2011).

1.2.2 Amyloid hypothesis

Amyloid hypothesis, together with tau, is one of the most cited theories about the causation of AD. This hypothesis starts with the altered cleavage of the amyloid precursor protein (APP). APP is an important protein in the plasma membrane. This altered cleaving is done by β-secretases (BACE1) and γ-secretases, and results in insoluble Aβ peptides. The Aβ peptides then form oligomers, which may interfere with signalling. Furthermore, these oligomers form plaques that lead to the damage and death of neurons (Tiwari *et al.*, 2019). APP is a type I intermembrane protein with a distinctive Aβ sequence of 40–42 amino acid; the longest APP isoform is 770 amino acids long. The APP is processed not only by already mentioned β- and γ-secretases, but also by α- and η- secretases (Nguyen, 2019). Under the action of these secretases, APP produces C-terminal fragment in three pathways. The non-amyloidogenic pathway produces, under the activity of α- and γ-secretases, neuroprotective fragments such as C-terminal fragment (CTF)-α, and the soluble ectodomain of APP-α (sAPPα). In the amyloidogenic pathway (Figure 5.), APP is cleaved by β-secretase into CTF-β, followed by γ-secretases into $A\beta_{42}$ (and some other fragments of different lengths). However, it is $A\beta_{42}$ that has the greatest propensity to polimerize and form plaques. The third pathway is an alternative pathway in which APP is cleaved by η-secretases (Fan *et al.*, 2020).

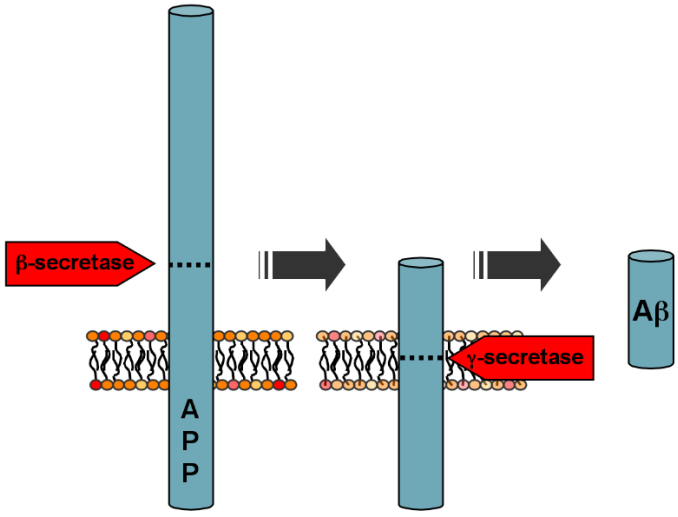

Figure 5. Amyloidogenic pathway showing the processing of APP, Aβ precursor (https://en.wikipedia.org/wiki/Amyloid_beta#/media/File:APP_processing.png)

In the form of toxic oligomers, Aβ may be causing mitochondrial damage, synaptic dysfunction, as well as inducing microglia and astrocytes, which in turn activate inflammatory and oxidative responses. This may lead to activation of apoptotic pathways. Furthermore, Aβ can activate tau protein kinase 1; this kinase can cause hyperphosphorylation of tau proteins and lead to the development of NFT (Fan *et al.*, 2020).

1.2.3 Tau hypothesis

Tau theory focuses on the hyperphosphorylation of tau proteins, which leads to the formation of NFTs that are found in the neuronal and glial cytoplasm and their processes (dendrites and axons). In normal environment, tau protein stabilizes microtubules composed of α and β tubulin (Tiwari *et al.*, 2019), although it is not the only protein that does so, but is supported in this role by other proteins as well (Šimić *et al.*, 2017). Tau itself is a product of alternative splicing of the *MAPT* gene. In normal conditions, tau is phosphorylated to some extent, but in the case where most of its phosphorylation sites are phosphorylated, it leads to hyperphosphorylation. Hyperphosphorylated tau leads to a problem in normal functioning of microtubules, and these tau proteins also start to form PHF, which in the end form NFT (Figure 6.). Accumulated evidence suggests that NFT lead to neurotoxicity and cell dysfunction. It has also been suggested that soluble tau protein can propagate, thus causing more harm that NFTs (Fan *et al.*, 2020).

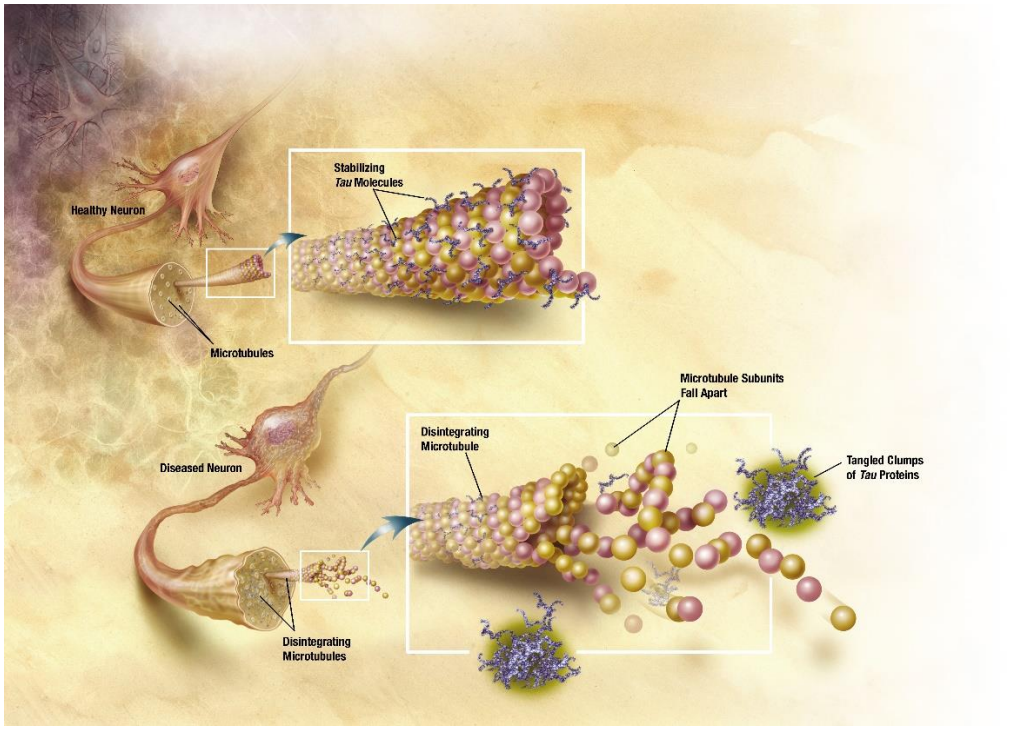

Figure 6. Changes in the phosphorylation of tau proteins that lead to the disintegration of microtubules and formation of NFT(https://en.wikipedia.org/wiki/File:TANGLES_HIGH.jpg)

Tau protein is a phosphoprotein and belongs to family of proteins called microtubule-associated proteins (MAP). These proteins play substantial roles in the stabilization of microtubules, the anterograde and retrograde cell transport, especially in neuronal cells, as well as proper function of mitochondria. In neurons, tau can be found in axons, but can migrate to dendrites if its structure and function are affected. The latter can cause aggregation of tau proteins, which leads to tauopathies, a group of neurodegenerative diseases such as frontotemporal dementia (formerly known as Pick's disease, its neuropathological substrate is termed frontotemporal lobar degeneration) (Tudorică *et al.*, 2017). Human tau protein comprises of 16 exons. Alternative splicing of the single *MAPT* gene gives rise to six major isoforms present in the human brain, mainly in neurons, but also in glial cells. These isoforms are 352 to 441 amino acids long and contain either none, one or two inserts on the amino terminus (0N, 1N, or2N) and also three or four microtubule-binding (MTB) repeats (3R or 4R) on the carboxyl terminus of the molecule (Šimić *et al.*, 2016, Tudorică *et al.*, 2017).

The amino (N-) terminus of the tau does not bind to microtubules and can interact with mitochondria and plasma membrane. This terminus is called projection domain. On the other hand, carboxyl (C-) terminus of the protein is called assembly domain, and this part of the protein interacts with microtubules. The third domain of the tau protein is called proline-rich domain, and is, as its name suggests, rich in proline. This domain serves as a target for proline-directed kinases. Another important thing to note is that N-terminus is acidic, while C-terminus is basic, making tau protein a dipole; this is important not only for microtubule interactions, but also for aggregation and folding of tau (Mietelska-Porowska *et al.*, 2014).

Phosphorylation is the main post-translational tau modification; the protein has many binding sites where this may occur, most of which are serine-proline and threonine-proline sites. However, phosphorylation is not the only post-translational process involved in tau modification: O-glycosylation is a process in which O-linked N-acetylglucosamine (O-GlcNAc) is added on serine or threonine residues (in the vicinity of proline). This supposedly plays a role in the degradation of tau proteins (Šimić *et al.*, 2016). Acetylation is another modification of tau proteins which seems to be protective against the polymerization and aggregation of tau. Hypoacetylation, however, at specific sites has been found in AD patients, which would suggest this might be a first step toward

eventual AD pathological changes. It is important to mention that acetylation at different sites of tau protein may very well have the opposite effect (Šimić *et al.*, 2017).

In AD, the cause of tau aggregates seems to be tau hyperphosphorylation; in these cases, tau is up to four times more phosphorylated than in normal brains without pathological changes. Hyperphosphorylated tau cannot bind to microtubules and leads to the formation of NFT (Tudorică *et al.*, 2017). This abnormal tau also differs in other properties such as higher molecular mass, it is insoluble, and also reacts to certain antibodies in a phosphorylation-dependent manner (Biernat *et al.*, 1992). It can also sequester not only normal tau, but also MAP1 and MAP2, other two neuronal MAP, and it can assemble into either PHF or straight filaments (SF) (Iqbal *et al.*, 2010).

Kinases are the most common enzymes that phosphorylate tau proteins; these enzymes can be divided into three groups and those are proline-directed protein kinases (PDPK), non-PDPK protein kinases, and tyrosine protein kinases (TPK); there are many enzymes in each group (Mietelska-Porowska *et al.*, 2014).

1.2.3.1 Spreading of tau pathological changes

Propagation of neurofibrillary changes in the brain can be divided in six stages, as it follows a pattern described by Braak and Braak (1991). These stages (Figure 7.) can be also observed in the spread of neuropil threads (NT), structures made of straight and paired helical filaments that represent hyperphosphorylated and accumulated tau in axons. Stages I and II are also known as transentorhinal stages, as this region is the first one to develop NFT, and they are mostly located in this region, with isolated NFT that can be found in regions like anterodorsal nucleus of the thalamus or CA1 area of hippocampus. In stage I, changes in the transentorhinal region are still considered mild. In stage II, the number of NFTs in transentorhinal region increases. Stages III and IV are called limbic stages; the entorhinal cortex is most affected at this stage, with hippocampus being moderately affected, and isocortex almost not involved at all in these changes. In stage III, entorhinal cortex becomes affected, ghost tangles (solitary structures seen after neuronal death that were previously present in neuronal cytoplasm) can also be detected. In stage IV, ghost tangles are present in both trans- and entorhinal regions and NFTs can also be found in the CA1 region of the hippocampus, as well as in the amygdala and anterodorsal thalamic nucleus. Stages V and VI are called isocortical stages; both hippocampus and isocortex are severely affected. In stage V, all regions of hippocampus are affected by NFTs, as is isocortex. In stage VI, all these areas are even more affected. All of these stages have different clinical presentations in AD patients that correspond to anatomical regions affected (Braak and Braak, 1991).

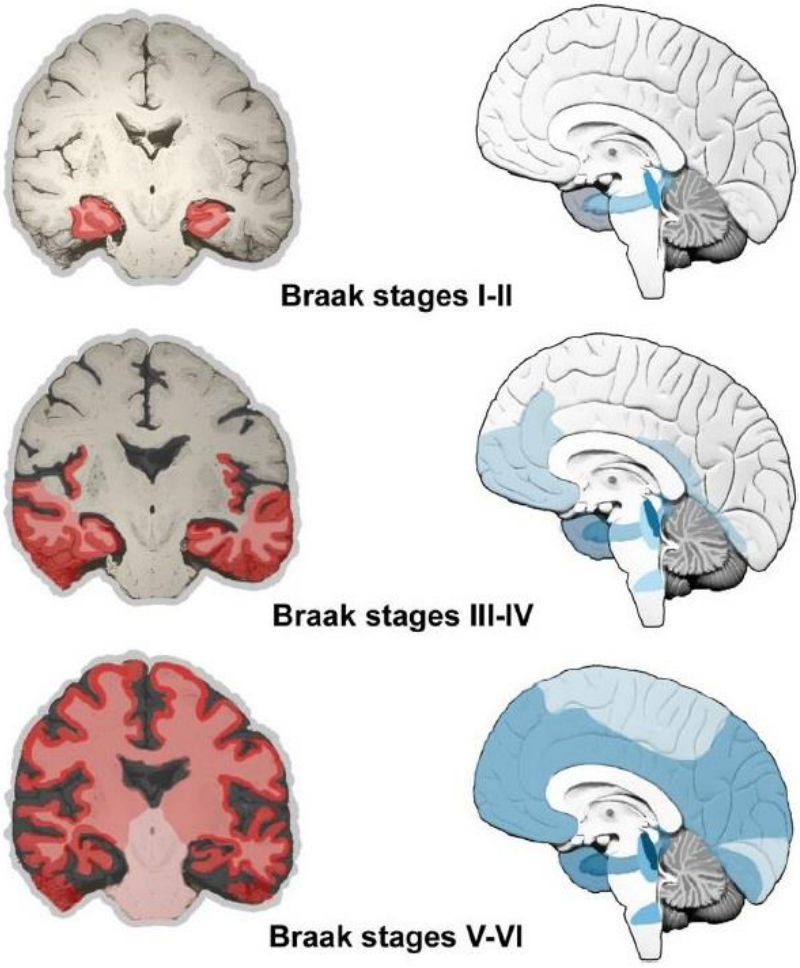

Figure 7. Braak stages of neurofibrillary propagation; from Šimić *et. al.*, 2017.

Based on these particular discoveries, mice models of AD have been developed, in order to better study AD. The transgenic mice (PS19 and PDAPP Tg hybrids) serve to study not only tau, but also Aβ hypothesis. Focusing on the tau theory, the model was characterized by T34 isoform of tau with one N-terminal insert and four microtubule binding repeats (1N4R) encoding the P301S mutation, under the control of mouse prion protein promoter. Antibody used to map the spread of tau proteins through mice brain was AT8, a monoclonal antibody which binds to abnormally phosphorylated tau on Ser202/Thr205 binding sites. Mice (PS19, PDAPP, and hybrids) were sacrificed at four, eight, and eleven months, and their brains were analysed using immunohistochemical methods; coronal sections 6 μm thick were used and sections were analysed at bregma levels -0.145, -2.055, and -2.88. The spread of tau proteins was compared to the Braak stages, and many similarities were found, despite the profound difference in human and murine brains. Stages in the mice brain were discerned as entorhinal/superficial neocortical stage (stage I/II), limbic/amygdala stage (stage III/IV), and deep neocortical/subcortical nuclei (stage V/VI). In PS19 mice models, the spread of tau followed the Braak model, although there some deviations were indeed noted: in mice, some layers of isocortex show NFTs as early as in stages I and II (opposed to Braak's stages II and III), and the same is true for amygdala and thalamic nuclei (changes appear in stages II and III, while in Braak's stages, these types of alterations were noted in stages III and IV). The most prominent difference, however, in murine models, is accelerated change in neocortical and subcortical regions. There was also one notable difference between PS19 mice model and a hybrid model: in hybrids, tau progression was accelerated. It was concluded that Aβ itself actually accelerates the formation of tau aggregates, while the opposite was not true (the presence of tau proteins does not affect Aβ) (Hurtado *et al.*, 2010).

More research using transgenic mice followed, focusing on the spread of abnormal tau proteins in mice brains. In one such study, tau protein (human P301S tau) was extracted from transgenic mice and then injected into brains of mice that express wild-type human tau, in order to observe possible impact this might have on spreading of the protein. The mice with wild-type protein do not form tau filaments, while P301S line does. This particular experiment showed that the injection of P301S tau did cause the formation of filaments (made of wild-type tau isoform) and also spreading of tau to the regions neighbouring the injection site (Clavaguera *et al.*, 2009). Apart from mice, rat models were also used. First study of this type successfully proved that brains of rat models undergo the same pathological changes when external tau protein is introduced. Furthermore, three different

tau extracts from human AD were used, and certain differences were noted, such as variations in spreading speed. However, perhaps the biggest importance of this study was that it proved rats were also great murine models for the investigation of pathological changes caused by tau protein (Smolek *et al.*, 2019).

It is exactly this type of tau propagation and spreading to neighbouring regions that prompted scientists to consider a possible prion involvement. Some evidence suggests that aggregates of not only tau proteins, but also Aβ, act similarly to prions (Figure 8.), which would allow these proteins to spread through the brain (Zhou, 2013).

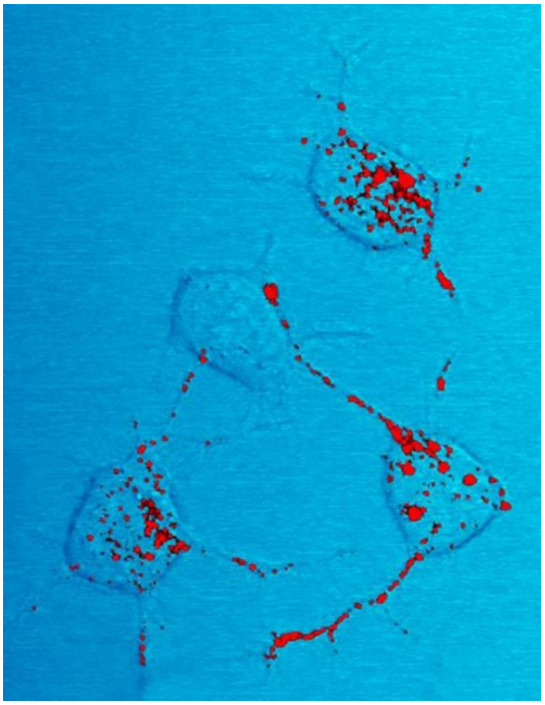

Figure 8. Red-stained prion proteins in neural tissue of a mouse infected with the scrapie disease (https://en.wikipedia.org/wiki/File:Scrapie_prions.jpg)

Prion diseases are also neurodegenerative disorders, characterized by prions (PrP^{C}), proteinaceous infectious particles (Kellett and Hooper, 2009). Prion disease can be sporadic (spontaneous), genetic, and acquired. In these diseases, PrP^{C} undergoes a conversion into PrP^{Sc}, which is an abnormal form of the protein. PrP^{Sc} then, acting as a template, converts other PrP^{C} proteins; this lead to the accumulation of PrP^{Sc} protein (Figure 9.), which then possibly leads to neurodegeneration (Geschwind, 2015). In AD, PrP^{C} has been identified as a cell surface receptor for Aβ proteins (Zhou, 2013).

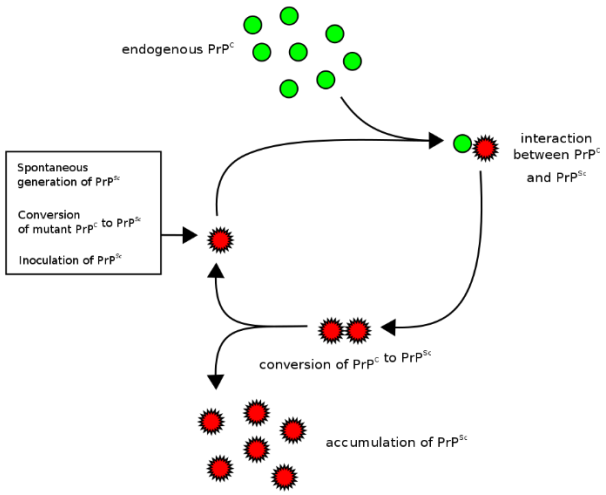

Figure 9. One of the possible models that explains the prion propagation (https://en.wikipedia.org/wiki/File:Prion_propagation.svg)

It has been suggested that in AD, PrP^C actually transduces and mediates toxic signals arising from Aβ proteins, specifically Aβ42 oligomers. In *in vivo* experiments on mice, it has been shown that mice lacking PrP^C did not show the impairment in spatial learning and memory while the ones that did have PrP^C did. However, PrP^C has also shown its protective functions, as it inhibits *N*-Methyl-d-aspartate (NMDA) receptors, which leads to the suppression of glutamate-mediated neuronal excitatoxicity, and more importantly, it interacts with BACE1, which is an APP cleaving enzyme; this results in the reduction of Aβ protein production (Zhou, 2013) (Kellett and Hooper, 2009). It would seem that PrP^C acts protective in non-AD brains, but mediates the $A\beta_{42}$ oligomers toxicity in the later stages of the disease (Kellett and Hooper, 2009).

When it comes to tau specifically, all of the proposed models of tau propagation practically mimic the prion models. The spread of tau proteins through the brain, which was described in detail in previous paragraphs, is very similar to proposed spread of prion proteins. Another similarity is the existence of both prion and tau strains: differently folded prions and misfolded tau proteins lead to distinctively different clinical pathologies (Ayers *et al.*, 2018). Experimental models in mice confirmed that tau acts as a prion, since it basically clones itself during propagation. The cell to cell spread and self-propagation could be enough to classify tau as a prion protein, although it lacks the “infectivity” of typical prions (Sanders *et al.*, 2014). Naturally, many more studies are needed in order to gain further insight into these intriguing experimental data.

1.2.4 Other hypotheses

There are many other theories and hypotheses about the cause of AD, such as cholinergic, oxidative, inflammatory, and environmental.

Cholinergic theory suggests that, because cholinergic pathways play an important role in cognition and memory, the degeneration of these pathways as well as the decline in concentrations of acetylcholine lead to the development of AD (Francis *et al.*, 1999).

Oxidative hypothesis is focused on the importance of oxidative processes in AD. This theory suggests that Aβ and tau plaques are a result of oxidative stress, mainly the imbalance between oxidants and antioxidants in the brain, which is vulnerable to this type of stress. The imbalance is on the side of the oxidants and can occur because of the increase in the free radicals, mainly reactive oxygen species (ROS). ROS can interact with many other molecules, such as nucleic acids, proteins, and lipids (Huang *et al*., 2016). Metals like copper and iron also play a role in the oxidative stress (they are redox transition metals); both are present in elevated levels in AD (Perry *et al*., 2002).

Inflammation hypothesis suggests that Aβ plaques and NFTs stimulate inflammation in the brain, and during the years, this inflammation only exacerbates the course of the disease (Akiyama *et al.*, 2000). In this inflammatory process, microglia secrete cytokines, reactive oxygen species, and nitric oxide (Greer, 2000). Basically, neuroinflammation in this case is a result of both Aβ and tau aggregates and it results in microglia activation, which in turn plays a role in the neuronal degeneration (Zotova *et al.*, 2010).

Environmental hypothesis focuses on various environmental factors that could play a role in the development of AD. Factors that could bare a risk were identified in a study and include air pollution (air containing nitrogen oxides, particulate matter, and ozone), metals such as aluminium, metalloids such as silicon and selenium, pesticides, solvents, and vitamin D deficiency (Killin *et al.*, 2016). Of all of these, aluminium has perhaps been studied the most, as it is abundant on the planet. Small amounts of aluminium are indeed enough to cause neurotoxicity in the brain, and it could indeed pass the blood-brain barrier (Tomljenovic, 2011).

2 Research goals

It has always been enigmatic what initiates the conversion of a highly soluble tau protein without defined secondary structure into insoluble β-sheet fibrils. Recently, numerous studies have shown that exogenously supplied pathological tau, including synthetic tau fibrils, can drive soluble tau into tangle-like inclusions in both cell culture systems and mouse models, implicating a seeding-recruitment process as well as cell to cell transmission of pathological changes as possible underlying mechanisms for the initiation and progression of tauopathies. As synthetic tau oligomers and tau fibrils can spontaneously enter neuronal and non-neuronal cells (Peng *et al.*, 2020) and recruit normal tau into pathological accumulations, the goal of this work was to further investigate some aspects of the tau protein behaviour in a rat model of AD.

The specific aims of this Master's thesis were:

- to study the propagation of tau protein in the rat brain at different time points after intracerebral administration of tau oligomers and synthetic tau fibrils

- to analyse possible similarities and differences in propagation of inoculated tau oligomers and tau fibrils

- to analyse which form of protein (tau oligomers or tau fibrils) causes faster tau spread and a greater range of pathological changes at different time points

3 Materials and methods

3.1 Samples

All samples were obtained by coronal sections of rat brains. Male Wistar rats, between three and four months of age, were divided into three groups: control (CTR), tau fibrils (TF), and tau oligomers (TO). Control group was intracerebrally administrated phosphate-buffered saline (PBS), TF group was administered synthetic tau fibrils, and TO group was administered tau oligomers; all animals received stereotaxical injections into the entorhinal cortex. In total, nine animals were sacrificed, and their brains preserved (at four, eight, and eleven months post-injection), with three sections (bregma -8.04, -6.84, -5.64) of each brain analysed, for a total of 27 sections. A section of a brain of a patient suffering from Alzheimer's disease was used as a positive control.

3.2 List of the materials and solutions

Composition and preparation of solutions

Phosphate-buffered saline

Phosphate-buffered saline (PBS) is prepared by mixing components listed below and distilled water to a final volume of 1 L. The pH of the buffer prepared is about 7.4.

First, add 800 mL of distilled water in the container, then add the components and adjust the pH.

- NaCl - 90 g
- KCl - 2 g
- Na_2HPO_4 - 12.3 g
- KH_2PO_4 - 2 g

- top up dH_2O to 1L

Citrate buffer

Citrate buffer is prepared by mixing citric acid monohydrate and distilled water to a final volume of 1 L of a pH of 6.0.

First, add 800 mL of distilled water in the container, then add the components and adjust the pH.

- Citric acid monohydrate – 2.1 g
- Adjust the pH with 2M NaOH

- top up dH_2O to 1L

Pre-treatment

Pre-treatment is prepared by mixing methanol, hydrogen peroxide, and distilled water.

- Methanol – 150 mL
- H_2O_2 – 150 µL
- dH_2O – 50 mL

(Hydrogen peroxide should be added just before we put slides into glasses with the pre-treatment.)

Blocking solution (5% BSA + 0.5% TRITON/PBS)

This solution is prepared by mixing BSA, TRITON X, and PBS, to a final volume of 25 mL.

- add TRITON X – 125 µL to 20 mL of PBS (using magnetic stirrer)
- slowly add 1.25 g of BSA
- top up dH2O to 25 mL
- filtrate into a new “Falcon” tube

DAB

In 5 mL of distilled water, dissolve 1 tablet of DAB, vortex the solution and then dissolve 1 tablet of hydrogen peroxide.

DAB neutralization solution

DAB neutralization solution is prepared by mixing sodium hypochlorite, NaOH, Na_2CO_3, and distilled water to a final volume of 1 L.

- sodium hypochlorite – 150 mL
- NaOH – 4 g/L
- Na_2CO_3 – 15 g

- top up dH_2O to 1L

Xylene, absolute ethanol, 96% ethanol and **70% ethanol** for de- paraffinization.

Primary, secondary, and **tertiary antibodies** for protein visualisation.

- Primary antibody should be diluted (1:100) in the blocking solution
- Secondary antibody should be diluted (1:200) with BSA 50% solution (which was beforehand diluted with PBS)
- Tertiary antibody is prepared by mixing 2 mL of diluted BSA + 10 μL of Avidin + 10 μL of Biotin (it takes around 30 minutes for Avidin and Biotin to bind, during which the tube should be periodically vortexed)

Histoclear and **histomount** for the overlapping of histological slides.

3.3 Microtome cutting

Using a microtome, slides were prepared for further analysis. Paraffined blocks, containing previously isolated and preserved rat brains, were carefully cut and then placed on the slides. Such coronal sections, 10 μm thick, were they dried for 24 hours.

3.4 Nissl staining

Nissl staining was used in order to choose three levels of coronal sections which were then used in the further research. The first step in the staining was the deparaffinization which was done by the series of rinses with xylene and alcohol (alcohol also works as a re-hydrating medium). Various percentages of alcohol were used, in a decreasing concentration (absolute, and 70%). After this, slides were rinsed in PBS for ten minutes.

Samples prepared in this way were then treated by the 0.5% solution of cresyl violet diluted in distilled water in a ratio of 1: 4, for up to five minutes. Afterwards, the slides were rinsed in distilled water, 70% alcohol (twice), absolute alcohol (twice, for three minutes), and xylene (twice). After they are dried, the slides are mounted using one drop of Histomount.

In this way, using light microscope, the three coronal sections were identified (bregma -8.04, -6.84, -5.64). These sections were then further researched by using immunohistochemical staining and analysis.

3.5 Immunohistochemistry

Immunohistochemical staining is used in order to visualize hyperphosphorylated tau proteins in the rat brain tissue. In this case, AT8 antibody was used as a marker for tau activation and spread.

3.5.1 Staining

The first step in the immunohistochemical staining is deparaffinization and rehydration of the samples, that was already described in the previous sub-chapter. The next step is the antigen retrieval (eliminating possible chemical modifications of the epitopes occurred during paraffinization) which was achieved by cooking the slides in the citrate buffer (in the microwave).

The slides were then rinsed in PBS and treated by the pre-treatment solution, in order to inactive peroxidase activity. The slides were once again rinsed in PBS.

The next step is blocking of the unspecified binding of the antibody. The blocking solution contains 5% BSA (bovine serum albumin) and 0.5% TRITON/PBS, which are supposed to make a thin layer on the surface of tissue and in that way block the unspecified binding. Before putting the blocking solution on the slides, all sections are circled by a PAP pen (hydrophobic solution; Sigma Aldrich),

which acts as a barrier that prevents blocking solution from dripping down. Slides with blocking solution are incubated on the room temperature for the 60 minutes in the wet chamber.

Primary antibody is a mouse antibody (TermoFisher, Phospho-Tau (Ser202, Thr205) Monoclonal Antibody (AT8),) and as such, was diluted in the blocking solution before use. The diluted ratio was 1:100. After blocking solution was carefully removed, all slides, apart from negative control, were covered with diluted primary antibody and incubated overnight at the temperature of +4 °C in the wet chamber. The negative control is actually a control of the antibody binding specificity.

The next day, the slides were rinsed in PBS. Secondary antibody (anti-mouse; Vectastain ABC kit, PK-4002) is diluted in BSA solution (previously diluted with PBS) in ratio 1:200. All slides, including negative control, were covered in the secondary antibody and then incubated for 60 minutes at the room temperature in the wet chamber. Afterwards, slides were rinsed in PBS.

Tertiary antibody (Vectastain ABC kit, PK-4002) was made as a mixture of diluted BSA, Avidin, and Biotin. After all slides were covered, they were again incubated for 60 minutes at the room temperature in the wet chamber. Afterwards, slides were again rinsed in PBS.

The visualisation of slides was made using DAB solution (3,3′-Diaminobenzidine; SIGMAFAST DAB); every slide was developed for ten minutes and then rinsed with PBS (the DAB solution was drained in the glass containing DAB neutralization solution). Apart from rinsing with PBS, the slides were also rinsed with distilled water.

After drying, the slides were moved into a basin with Histoclear solution and afterwards covered with one to two drops of Histomount (Poly-Mount) and cover slips.

3.5.2 Slide microscopy and photographing

All slides were photographed by using an invert microscope (Olympus).

3.5.3 Analysis using FIJI software

FIJI software was used in the analysis and assessment of all photographed slides. Using this software, every brain region necessary for the research was carefully marked, measured, and phosphorylated tau proteins (represented in the photographs as a violet-coloured dots) were counted; this information was then used in the statistical analysis.

The regions, on every of the three sections were: hippocampal cortex and entorhinal cortex. As a help with marking the regions, The Rat Brain in Stereotaxic Coordinates, Sixth Edition by George Paxinos and Charles Watson was used.

4 Results

The sections analysed were divided into the three groups: control (CTR), tau fibrils (TF), and tau oligomers (TO) group. Three coronal sections from each brain were analysed (Figure 10.). The CTR group were sham operated animals who received PBS injections, while TF group was injected with tau fibrils, and TO group was injected with tau oligomers. Altogether, 27 immunohistochemical sections were photographed and analysed using the FIJI software, an open-source platform for biological analysis (https://imagej.net/software/fiji/). As the two main regions, hippocampus and entorhinal cortex, were analysed in the right hemisphere, this resulted in 54 separate structures evaluated by using a semiquantitative method.

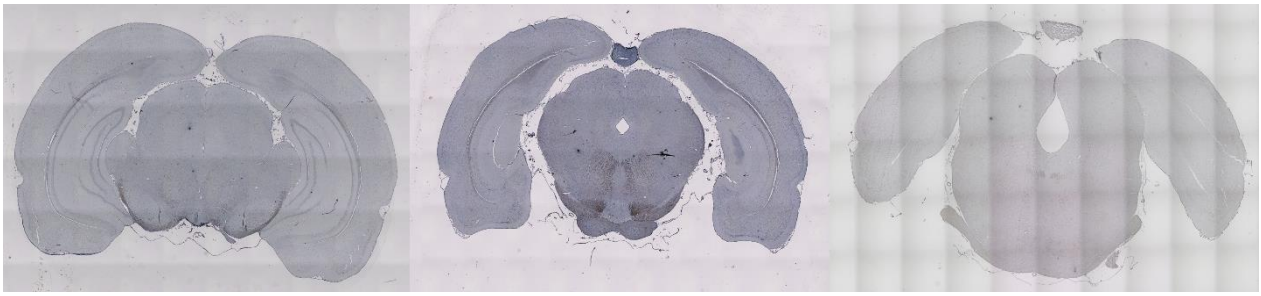

Figure 10. Three coronal sections of a rat brain (from left to right: -5.64, -6.84, and -8.04 mm from bregma) taken under the inverted microscope.

Immunostaining with AT8 antibody was successful in all sections. AT8-immunoreactivity (AT8-ir) was easily distinguished in the pictures due to its dark colouring (Figure 11.). AT8 antibody is antibody used to mark phosphorylated tau proteins, because it binds to phosphorylated Ser202/Thr205 epitope of tau, the binding site considered to be the most significant in the course of AD (Hurtado *et al.* in 2010).

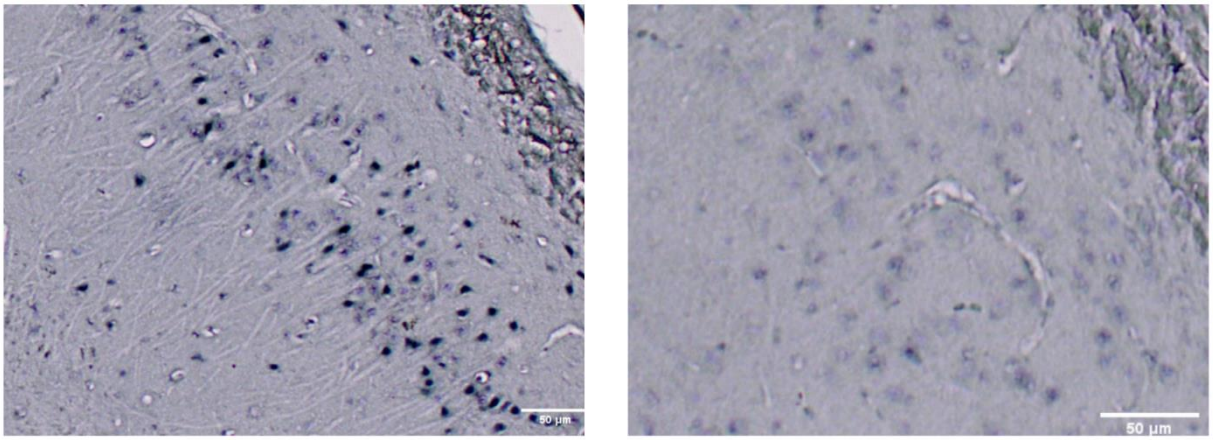

Figure 11. Comparison of the same hippocampal area in two different immunohistological slides, clearly showing the difference in staining intensity. Left figure shows a coronal brain section from an animal of the TF group, while the right figure shows a coronal section of a brain from the animal of TO group.

In this research, every tau tangle that was deemed as a pathological change was marked with a dot (Figure 12.) and the results of every analysed area were recorded.

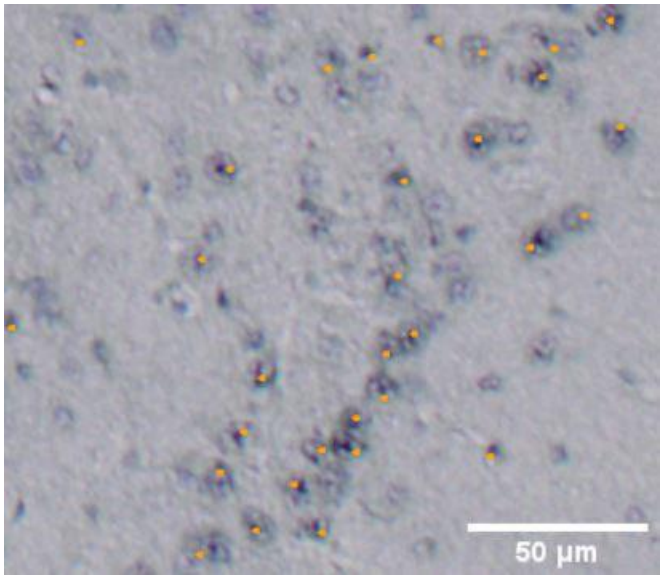

Figure 12. An example of marking hyperphosphorylated tau proteins in FIJI software.

All results were then quantified using a scoring system described in Table 1., in order to evaluate and score tau pathological changes of each brain section that was analysed in the research.

Table 1. Semiquantitative scoring system.

Immunoreactivity	Scoring	Number of immunoreactive cells
None	0	0
Rare	+ / -	1-200
Moderate	+	201-500
Abundant	+ +	>501

The comprehensive results of applying this scoring system are presented in Table 2., where results are grouped, and scores are colour coded for easier navigation.

Table 2. Results of semiquantitative analysis separated into three groups (CTR, TF, TO), also showing the age of animals (4 m, 8 m, 11 m), and coronal section analysed (-5,64, -6.84, -8.04). Two areas are analysed, hippocampus (HC) and entorhinal cortex (EC), using the scoring system described in Table 1.

Cross section	Area	Score	Cross section	Area	Score	Cross section	Area	Score
CTR 4m -5.64	HC	+-	TF 4m -5.64	HC	+	TO 4m -5.64	HC	+-
CTR 4m -5.64	EC	+-	TF 4m -5.64	EC	++	TO 4m -5.64	EC	+-
CTR 4m -6.84	HC	+-	TF 4m -6.84	HC	+	TO 4m -6.84	HC	+-
CTR 4m -6.84	EC	+	TF 4m -6.84	EC	+	TO 4m -6.84	EC	+-
CTR 4m -8.04	HC	+-	TF 4m -8.04	HC	+	TO 4m -8.04	HC	+-
CTR 4m -8.04	EC	++	TF 4m -8.04	EC	+	TO 4m -8.04	EC	+-
CTR 8m -5.64	HC	+	TF 8m -5.64	HC	+	TO 8m -5.64	HC	+-
CTR 8m -5.64	EC	+	TF 8m -5.64	EC	+	TO 8m -5.64	EC	+-
CTR 8m -6.84	HC	+-	TF 8m -6.84	HC	++	TO 8m -6.84	HC	+-
CTR 8m -6.84	EC	+	TF 8m -6.84	EC	++	TO 8m -6.84	EC	+-
CTR 8m -8.04	HC	+-	TF 8m -8.04	HC	+-	TO 8m -8.04	HC	+-
CTR 8m -8.04	EC	+	TF 8m -8.04	EC	+	TO 8m -8.04	EC	++
CTR 11m -5.64	HC	+-	TF 11m -5.64	HC	+-	TO 11m -5.64	HC	+-
CTR 11m -5.64	EC	+-	TF 11m -5.64	EC	+	TO 11m -5.64	EC	+
CTR 11m -6.84	HC	+-	TF 11m -6.84	HC	+-	TO 11m -6.84	HC	+-
CTR 11m -6.84	EC	+-	TF 11m -6.84	EC	+	TO 11m -6.84	EC	+
CTR 11m -8.04	HC	+-	TF 11m -8.04	HC	+	TO 11m -8.04	HC	+-
CTR 11m -8.04	EC	+	TF 11m -8.04	EC	++	TO 11m -8.04	EC	+

The results clearly indicate heightened pathological changes in the TF group, which were not present in the TO group. Actually, the TO group seemed to have lower scores of pathological changes in comparison to the control group (Figure 13.).

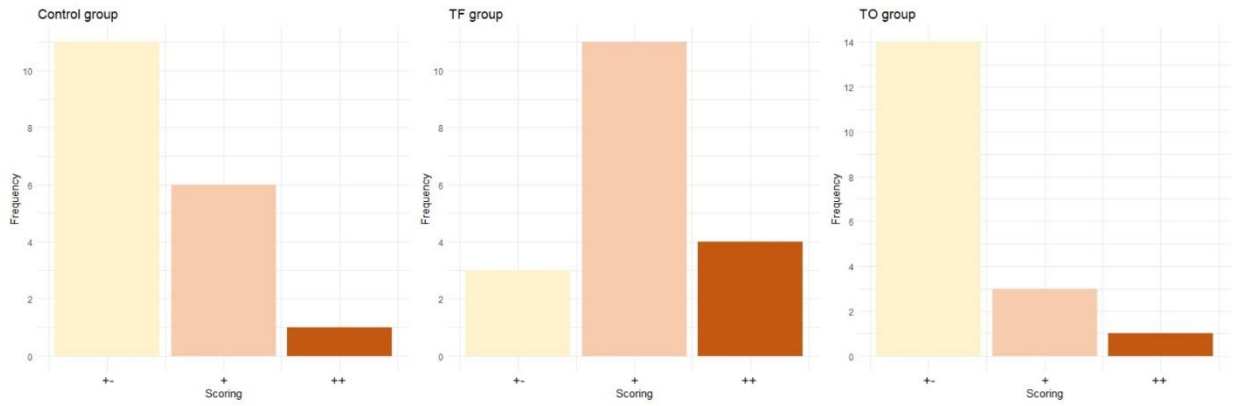

Figure 13. Barplots showing total frequency of scores (rare: +-, moderate: +, abundant: ++) for each experimental group (CTR, TF, TO). The highest amount of tau pathological changes is displayed in the TF group and the lowest in the TO group.

When comparing the possible spread of pathological tau changes from the entorhinal cortex to hippocampus, the propagation was indeed present in the TF group, where pronounced changes in the hippocampal formation were noted, while such changes in the other two groups were only barely present (Figure 14.).

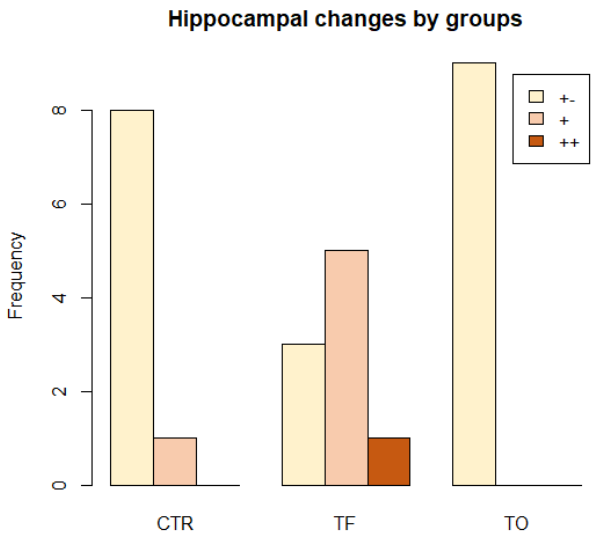

Figure 14. Changes in the hippocampus in each experimental group (CTR, TF, TO); scores of the TF group show changes in the hippocampal formation, while these changes are barely present in the CTR group and not present in the TO group.

There was also a slightly larger extension of pathological changes displayed in the brains of older animals in the TO group. This trend was not noted in the CTR and TF groups. There were no significant differences in other pathological changes found (Figure 15.).

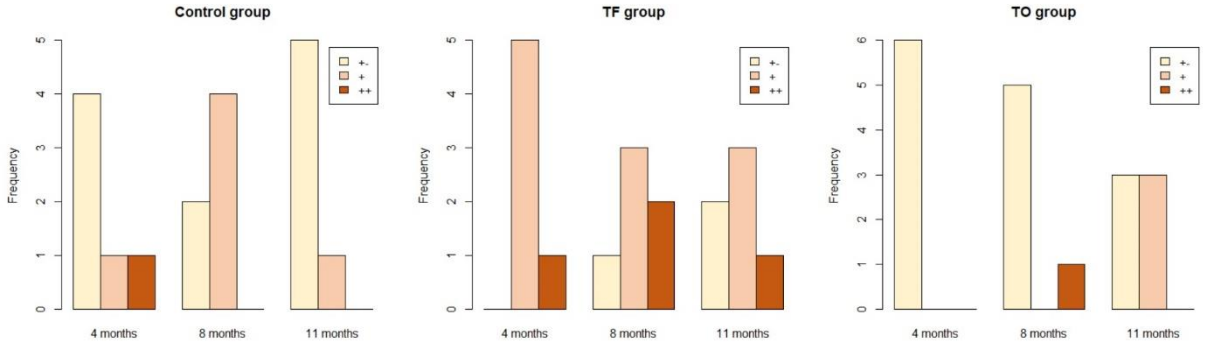

Figure 15. Barplots showing total frequency of scores (rare: +-, moderate: +, abundant: ++) for each group (CTR, TF, TO) and each time point (4 months, 8 months, 11 months) in the groups. There are no significant pathological differences in the brains of older animals in CTR and TF groups (pathological changes in all age groups are similar), while the TO group displays an observable trend in the increase of pathological changes with the age of animals.

5 Discussion

In this research, nine, previously paraffined rat brains were analysed, and they were further sectioned into three coronal sections each (bregma -8.04, -6.84, -5.64), with a microtome, resulting in 27 pictures. In each picture, two areas were analysed, entorhinal cortex and hippocampus. The brains originated from three different groups of animals: control group (sham, injected with PBS), TF group (injected with human tau fibrils), and TO group (injected with human tau oligomers); all animals were injected into right entorhinal cortex.

The main finding of this work is that animals from the TF group have shown the greatest score of pathological changes and spread of tau fibrils (the propagation from entorhinal cortex to hippocampus was noted), while such changes did not occur in the CTR or TO groups. These findings suggest that tau fibrils may be the true culprit in AD as they spread from their originating point and cause specific pathology in tissues they spread to. Furthermore this spreading follows characteristic distribution pattern which allows for differentiation of Braak stages (Braak and Braak, 1991). The spread of tau fibrils would suggest that fibrils indeed follow a pattern of prion-like propagation, and that the spread is consistent with the model of cell to cell transmission. This suggests that tau could be classified as a prion protein, since it propagates with high fidelity and encodes self-catalysing information. However, there is no evidence so far that tau is infectious in the same sense as prions (Sanders *et al.*, 2014).

On the other hand, the results obtained in the TO group lead to a conclusion that tau oligomers do not spread and do not cause tau pathological changes *per se*. However, these results are at odds with other findings about tau oligomers. According to some studies, tau oligomers, in fact, seem to be toxic tau forms and it has also been suggested that they may trigger pathological changes in the brain which ultimately lead to AD. Still, precise mechanisms by which tau oligomers trigger these events are unknown (Shafiei *et al.*, 2017; Niewiadomska *et al.*, 2021).

Only entorhinal cortex and hippocampus were analysed in this research, however, adding neocortex to the list of areas needed for the analysis could provide a further insight into the spread of tau pathological changes in rat brains, especially since that area was indeed analysed in a similar research based on mice brains (Hurtado *et al.*, 2010).

Apart from the propagation, one of the goals of this research was to analyse which form of protein causes a greater range of pathological changes at different time points. The brains of older animals in the TO group displayed slightly larger extension of pathological changes, however, this was expected to happen in all groups. Moreover, the pathological changes in the TO groups were so miniscule in general when compared to other two groups, that they are almost negligible. One possible reason for these results could be that all of the animals were sacrificed too young and further experiments should in all likelihood include longer time spans between animal sacrifices.

The main limitations of this study were a small sample size and an inherent variability of the immunohistochemical method (some slides were lighter coloured than the others, which was also noted in different shades of the so called "dots" that were counted towards the result). Further research should therefore include a larger sample size.

Furthermore, due to the fragility of immunohistological specimens, some breakage was noted in areas necessary for the analysis which could very well affect the results. Another issue faced while analysing the results was that some pictures obtained from the microscope were not as sharpened as they probably should have been. All of the aforementioned predicaments could have affected the analysis and with it, the final results of the research.

In this research, immunostaining with AT8 antibody that binds to phosphorylated Ser202/Thr205 epitope of tau was used with success, but future studies could benefit from including other tau antibodies, such as PHF-1, which is also known to yield high quality results (Moloney *et al*., 2021). Anti-tau antibodies that could also prove valuable include AT270 (recognizes PHF-tau Thr181), AT180 (recognizes PHF-tau Thr231), and T14 (recognizes human tau) (Hurtado *et al.*, 2010). Additional antibodies worth considering are Alz-50, MC-1, CP13, pS396, TauC3, GT-38, Ab39, and MN423, all of which are used to visualize various maturity stages of NFT (Moloney *et al*., 2021). These antibodies should be carefully chosen based on their immunoreactivity in target samples.

Other staining options, beside immunostaining, could also possibly yield fruitful results, such as fluorescent stains (thiazin red, thoflavin-S), Bielschowsky silver staining (which was used in the very first case of AD diagnosis), haematoxylin and eosin (known as H&E stain), and Congo red. However, these staining options might not cover the variety of NFT maturity levels in a way immunostaining does (Moloney *et al*., 2021).

In order to choose the best methods and possible antibody, it would be prudent to include a longer initial preparation phase in future research, both to compare current literature and to test out the staining methods on the samples. In that way, the most suitable method can be carried out later in the research.

6 Conclusions

Despite the small sample size and inherent variability of the immunohistochemical method, the results obtained through this work convincingly showed clear differences between the neuropathological changes and propagation of tau fibrils and tau oligomers. Whereas tau fibrils were found to cause characteristic tau pathological changes and also to spread from the entorhinal cortex to hippocampus, the inoculated tau oligomers did not induce such changes. The age of animals generally had no particular influence on tau pathological changes and spread. To be able to evaluate pattern of tau pathological changes and spread of the neurofibrillary changes more accurately, future studies should include a larger number of samples and a more rigorous quantification method.

7 Literature

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7.1 Online sources

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Curriculum vitae

I was born in 1993 in Varaždin, where I finished high school. In 2014 I started a BSc programme in Biology and in 2018 a MSc programme in Molecular Biology, both at Faculty of Science, Department of Biology, in Zagreb. During my studies, I was serving as an editor-in-chief of In Vivo student journal and an editor of Gyrus Journal, a student journal of neuroscience and I was active in the organizing committees of NeuRi – Student Congress of Neuroscience and SiSB - Student Symposium of Biology and Life Sciences. My special areas of interest are neurodegeneration and neuroinfectology. In my free time, I enjoy caving, knitting, and playing lacrosse.