CHARLES UNIVERSITY

Faculty of Science

Study program: Animal Physiology

Branch of study: Animal Physiology

Mgr. Lenka Hromádková

Tau protein, a biomarker of Alzheimer´s disease: *in vitro* phosphorylation and tau-reactive antibodies characterization.

Tau protein, biomarker Alzheimerovy choroby: *in vitro* fosforylace a charakterizace tau reaktivních protilátek.

Doctoral thesis

Supervisors: Prof. RNDr. Zuzana Bílková, Ph.D. (supervisor from 2016 to 2018)

RNDr. Daniela Řípová, CSc. (supervisor from 2012 to 2016)

Prague, 2018

The submitted dissertation contains the results obtained during my doctoral study under the supervision of RNDr. Daniela Řípová, Ph.D. at Laboratory of Biochemistry and Brain Pathophysiology and AD Center in Prague Psychiatric Centre (Prague, CZ), from 2015 at Research program of Experimental Neurobiology at National Institute of Mental Health (Klecany, CZ), and under the supervision of Prof. RNDr. Zuzana Bílková, Ph.D. at Department of Biological and Biochemical Sciences, Faculty of Chemical Technology at University of Pardubice (Pardubice, CZ).

This work was supported by Czech Science Foundation (project GACR P304/12/G069) and EU project NADINE (No. 246513).

Declaration:

I hereby declare that I have written this thesis independently, and all resources, as well as co-authors, were properly indicated. I also declare that I did not submit this thesis or a part of it, to obtain any other academic degree.

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.

……

Prague (Praha), 2018 Mgr. Lenka Hromádková

I would like to express my gratitude to my supervisor Prof. RNDr. Zuzana Bílková, Ph.D. for her guidance during my doctoral studies and that she enables me to perform a considerable part of my experimental work in a very friendly atmosphere at Department of Biological and Biochemical Sciences at the University of Pardubice. I would like to thank my previous supervisor RNDr. Daniela Řípová, Ph.D. for the opportunity to become a member of AD research group at Prague Psychiatric Centre and at National Institute of Mental Health from 2015. My sincere thank also belongs to Doc. MUDr. Aleš Bartoš, Ph.D. who was my supervisor specialist and to Mgr. Barbora Jankovičová, Ph.D. who helps me to learn how to formulate hypotheses and to set experimental workflow especially in the beginning of my research work. I also very much appreciate the collaboration with Mgr. Rudolf Kupčík from University of Pardubice and Mgr. Michala Krestová, Ph.D. from National Institute of Mental Health.

I would also like to thank my colleagues from National Institute of Mental Health and the University of Pardubice for their help, stimulating discussions and valuable advice.

My great thanks go also to my family and close friends who always support me to fulfill my dreams.

LIST OF CONTENTS

ABSTRACT

Tau protein, a microtubule-associated protein localized in axonal projections of neurons, is a key molecule in the pathology of Alzheimer´s disease (AD), the most common cause of dementia in the elderly population. Tau belongs to the group of natively unfolded proteins without globular structure and is prone to numerous posttranslational modifications (PTMs). Under pathological conditions, abnormal PTMs and misfolding of tau protein occurs and leads to oligomerization and aggregation into paired helical filaments forming neurofibrillary tangles, the histopathological hallmark of AD.

Currently available drugs applied in AD treatment can only slow the disease progression and those, which halt the AD-specific neurodegenerative processes, are still missing. Very promising and evolving therapeutic approach is immunotherapy, and even immunomodulation by administration of intravenous immunoglobulin (IVIG) products, a reservoir of natural antibodies from the plasma of healthy donors, has been already tested. The discovery of naturally occurring antibodies directed to tau (nTau-Abs) in body fluids of both AD and healthy subjects and their presence in IVIG begin the investigation of their therapeutic potential. Considering a wide range of possible modifications of tau and of various tau species (oligomers, aggregates etc.), the characterization of nTau-Abs is crucial step in understanding their physiological role and possible involvement in AD pathogenesis.

The main project goal was to isolate natural tau-reactive Abs from the plasma of AD patients, healthy controls, and IVIG product and compare them. Differences in IgG subclass distribution, avidities, and reactivity with various tau protein forms among these tau-reactive antibodies obtained from AD and healthy controls were revealed and discussed.

Phosphorylation is the most studied PTM of tau significantly participating in the modulation of its function and interactions. Abnormal phosphorylation is tightly connected with alterations of tau biology associated with the formation of neurotoxic tau species and aggregates. Thus, the second aim of the project was to prepare tau protein phosphorylated at residues specific for AD in high purity, which could further serve as a model protein in sensitive immunoassays applied with natural tau-reactive antibodies studies. Kinase-loaded magnetic beads prepared and characterized in our lab were applied for sequential *in vitro* phosphorylation of tau.

The thesis gives an overview about the biology of tau protein, enzymes involved in its (hyper)phosphorylation or truncation, and naturally occurring antibodies directed to tau as well as contains an experimental part composed from four key publications summarized our basic research regarding tau properties, phosphorylation, and its naturally occurring antibodies.

ABSTRAKT

Tau protein je s mikrotubuly asociovaný protein, který se nachází v axonech neuronů. Je klíčovou molekulou podílející se významně na patogenezi Alzheimerovy nemoci (AN), která je nejčastější příčinou stařecké demence. Tau patří do skupiny tzv. přirozeně nesbalených proteinů postrádajících globulární strukturu a vysoce náchylných k post-translačním modifikacím. Za patologických podmínek je tau protein abnormálně modifikován a chybně sbalován, což v důsledku vede k jeho oligomerizaci a agregaci do párově helikálních filament, které jsou základní jednotkou neurofibrilárních klubek, histopatologických útvarů typických pro AN.

Současné léky používané v léčbě AN neurodegenerativní procesy pouze zpomalují a lék zcela zastavující vlastní rozvoj nemoci stále chybí. V současnosti je velmi slibným a rozvíjejícím se terapeutickým přístupem imunoterapie. Testována je také imunomodulace pomocí intravenózních imunoglobulinových preparátů (IVIG), které obsahují velké množství přirozených protilátek běžně se vyskytujících u zdravé populace. Objevení přirozeně se vyskytujících protilátek reaktivních s tau proteinem (nTau-Ab) v tělních tekutinách u pacientů s AD, zdravých kontrol, i jejich přítomnost v IVIG preparátech odstartovaly snahy lépe pochopit jejich úlohu u AN a jejich potenciální terapeutické využití. Vezme-li se v úvahu, že tau protein podléhá mnoha modifikacím a existuje v různých formách (oligomery, agregáty atd.), je nezbytné nejprve tyto protilátky lépe charakterizovat a definovat jejich zapojení v patogenezi AN.

Hlavním cílem projektu proto bylo tyto protilátky izolovat z plazmy pacientů s AD, zdravých kontrol a IVIG preparátů a porovnat jejich vlastnosti a reaktivitu. Byla u nich zjištěna různá míra rozdílů v distribuci IgG podtříd, aviditě a reaktivitě s několika formami tau proteinu.

Fosforylace je ve vztahu ke správné buněčné funkci a interakcím tau proteinu jeho nejvíce studovanou post-translační modifikací. Abnormální fosforylace úzce souvisí s poruchami v biologii tau proteinu, protože má za následek tvorbu toxických tau forem a agregátů. Dalším cílem projektu byla příprava fosforylovaného tau proteinu ve vysoké čistotě, aby se mohl následně využít v imunoanalytických metodách pro studium charakteru a účinku nTau-Ab. Pro tento účel byly připraveny magnetické částice s navázanými kinázami a optimalizovány podmínky pro postupnou fosforylaci tau vícero kinázami.

První část dizertační práce obsahuje rešerši recentních poznatků o biologii tau proteinu, enzymech podílejících se na jeho (hyper)fosforylaci nebo krácení a také o výskytu a významu přirozeně se vyskytujících tau-reaktivních protilátek. V druhé části práce jsou formou 4 odborných článků shrnuty výsledky a závěry vlastního výzkumu zaměřeného na charakterizaci a vlastnosti tau proteinu jako významného biomarkeru AN a také na průkaz a charakterizaci tau-reaktivních protilátek nacházejících se v lidské plazmě.

KEYWORDS

Intravenous immunoglobulins; magnetic particles; natural autoantibodies; tau protein; posttranslational modifications; phosphorylation.

KLÍČOVÁ SLOVA

Intravenózní imunoglobuliny; magnetické částice; přirozené autoprotilátky; tau protein; post-translační modifikace; fosforylace.

LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
Aβ	amyloid beta
AEF	asparaginyl endopeptidase
ApoE	apolipoprotein E
ATP	adenosine triphosphate
AD	Alzheimer´s disease
CaMK	Ca2+/calmodulin-dependent protein kinase
Cdk	cyclin-dependent kinase

CK	casein kinase
CNS	central nervous system
DYRK	dual-specificity tyrosine-regulated kinase
ERK	extracellular signal-regulated kinase
GSK3	glycogen synthase kinase 3
IDP	intrinsically disordered
Ig	immunoglobulin
IVIG	intravenous immunoglobulin
JNK	c-Jun amino-terminal kinase
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
MARK	microtubule-affinity regulating kinase
MCI	mild cognitive impairment
MT	microtubule
MTBD	microtubule binding domain
nAb	naturally occurring antibody
nTau-Ab	naturally occurring antibodies directed to tau protein
NFT	neurofibrillary tangle
NPDPK	non-proline-directed protein kinase
PDPK	proline-directed protein kinase
PHF	paired helical filament
PK	protein kinase
PhK	phosphorylase kinase
PNS	peripheral nervous system
PP	protein phosphatase
PSA	puromycin sensitive aminopeptidase
PTM	posttranslational modification
TK	tyrosine kinase
TTBK	tau-tubulin kinase

INTRODUCTION

Alzheimer´s disease

As populations age, due to increasing life expectancy, the prevalence of age-associated neurodegenerative diseases has markedly increased. It is estimated that 46.8 million people worldwide living with dementia and it is assumed that this number increases almost threefold to 131.5 million in 2050 (http://www.alz.co.uk/research/world-report-2016).

The most common cause of dementia in mild-aged and elderly population is Alzheimer´s disease (AD). But dementia with all clinical symptoms that affect a person' ability to perform everyday activities is the final stage of AD progression which is preceded by two other clinical AD stages. The pre-clinical phase is characterized by some AD pathomorphological changes in brain tissue (depositions of amyloid beta (Aβ) peptides etc.) but individuals in this stage are cognitively normal. The second one is a prodromal phase of AD, referred to as mild cognitive impairment (MCI), characterized by the onset of the earliest cognitive symptoms including memory dysfunctions and other cognitive domains impairments which gradually shift to dementia, the third phase of AD (Jack et al., 2010).

Besides the gradual clinical decline, pathological processes leading to irreversible detrimental changes in AD brain also occur progressively, however, are detectable decades before the earliest clinical manifestation (Fig 1). The main two histopathological hallmarks occurring in AD brain tissue are represented by characteristic extracellular senile plaques composed of aggregated amyloid beta peptides (Aβ) and intraneuronal neurofibrillary tangles (NFTs) containing abnormally modified molecules of tau protein (Ittner and Gotz, 2011). Neurodegenerative changes manifested by gliosis and neuronal loss in cortical and subcortical regions leading to brain atrophy are also significant features observable in post-mortem brains of AD patients (Serrano-Pozo et al., 2011).

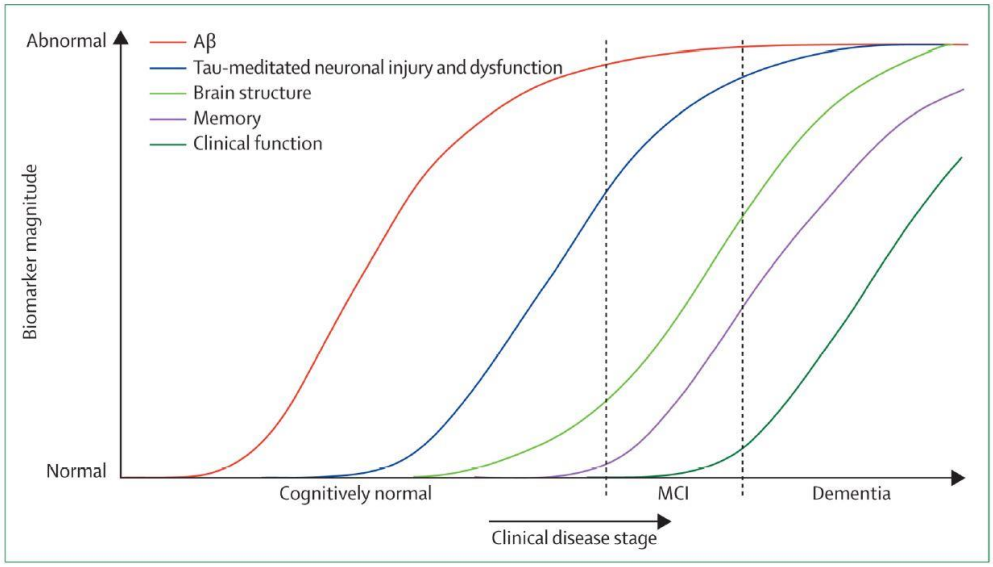


Figure 1: *Model of development of clinical manifestations and dynamics of biomarkers in time (Aβ as Aβ42 in cerebrospinal fluid or PET amyloid imaging, tau-mediated neuronal injury identified from tau levels in cerebrospinal fluid or fluorodeoxyglucose-PET imaging, brain structure based on structural MRI), adapted from (Jack et al. 2010)*

Despite the fact that AD affects a considerable part of the aging population and is ultimately fatal, neither definitive antemortem diagnostic method nor cure, which halts the disease progression, is currently available. AD is likely a multifactorial disease without a specific etiological agent and the pathogenesis has not yet been fully understood, thus more precise diagnostic biomarkers and specific drugs are still missing. Nevertheless, as indicated extensive scientific literature, several genetic risk factors and environmental factors playing role in pathogenesis and progression of AD have been described. Familial early-onset AD forms are associated with mutations in genes of proteins involved in the production of neurotoxic Aβ species, presenilin1/2 and amyloid precursor protein (Bateman et al., 2011). The late-onset AD that accounts for more than 95% cases is a sporadic form. Several genes are also associated with more severe pathological features in sporadic AD forms, for instance, carriers of the allele for ApoE4 have worse Aβ clearance mechanism than carriers of ApoE3 alleles (Laws et al.,

2003). Besides hereditary predispositions, the most discussed environmental factors connected with sporadic AD forms include metal ions accumulation (Wang and Wang, 2017), diet and malnutrition (Maruszak et al., 2014), brain trauma injury (Lye and Shores, 2000) and infectious agents (Itzhaki et al., 2016, Ghannad et al., 2016).

Also, many hypotheses of early events occurring in the development of AD have been suggested (reviewed by Armstrong, 2013). In the early nineties, Hardy and Higgins formed amyloid cascade hypothesis, the most dominant model of the molecular pathology of AD (Hardy and Higgins, 1992). This hypothesis accepts excessive depositions of Aβ peptides as a causative agent of AD pathology where hyperphosphorylated tau accumulation, neuronal loss, and cognitive decline are consequences resulting from the occurrence of toxic Aβ species (Selkoe and Hardy, 2016). A different, also very influential, hypothesis of AD initial pathological events was proposed in 2004 by Swerdlow and Khan as a mitochondrial cascade hypothesis. Mitochondrial dysfunction related to decreasing of ATP production and to increased oxidative stress leads to impaired protein clearance mechanisms, activation of the innate immune system and total imbalance of neuronal homeostasis with all the fatal consequences for post-mitotic neurons (Swerdlow and Khan, 2004). Also, defects in autophagy pathways, including mitophagy, and lysosomal proteolytic failure have been discussed in relation to neurodegenerative changes in AD. The underlying mechanisms of observed abnormalities in autophagy that are gradually revealed have a huge impact on new therapeutic strategies (Nixon and Yang, 2011). More suggested theories based on various key molecules explaining AD neurodegenerative events include, among others, cerebrovascular damage, degeneration of anatomical pathways as in case of cholinergic hypothesis (Francis et al., 1999), GSK3 hypothesis (Hooper et al., 2008), tau hypothesis (Maccioni et al., 2010) etc. The list of theories explicative molecular basis and mechanisms of AD pathophysiology is far from complete. All these hypotheses have been formed to better understand the molecular basis of AD initial events in an effort to find effective therapeutic tools, but a lot of questions associated with the concept highlighting one specific molecular factor has to be answered.

To date, there is a predominant view that a sum of many aspects, including genetic risk factors, environmental factors, and various altered molecular mechanisms contribute to AD in varying degrees and the pathophysiology is a consequence of more complex interrelated events.

Tau protein, a biomarker of Alzheimer´s disease

Tau protein is a key component of one of the histopathological hallmarks occurring in affected areas of AD brains called neurofibrillary tangles (NFTs) and is therefore investigated as a protein involved in disease mechanism as well as a potential diagnostic and therapeutic target of AD.

Tau protein was the first member of microtubule-associated proteins (MAPs) family described forty years ago (Weingarten et al., 1975). Tau protein is a heat stable factor essential for axonal microtubules' assembly promoting and stabilizing. Since its discovery in 1975, tau biochemical characterization, its biological function and distribution as well as the gene cloning and isoforms characterization followed and were successfully uncovered by several research groups in the last century (e.g.: Cleveland et al., 1977a, b; Binder et al., 1985; Drubin and Kirschner, 1986; Neve et al., 1986; Lee et al., 1988; Goedert et al., 1989; Goedert and Jakes, 1990). In a parallel with a deeper understanding of tau protein structure and properties, tau protein was identified as a protein subunit of NFTs localized in AD brain tissues by antibody reactivity (e.g.: Delacourte and Defossez, 1986; Wood et al., 1986). AD and other neurological disorders manifesting with a variety of clinical syndromes are characterized by tau deposition in the brain and are collectively known as tauopathies (Kovacz, 2015; Lebouvier et al., 2017). Taken together, tau is a protein of major interest and to better understand its role under both physiological and pathological conditions is an essential step in research of AD and tauopathies in general.

In the following introduction text, I attempt to briefly summarize the current knowledge about tau protein, including its structure, functions, and modifications. Also, the current knowledge about naturally occurring tau-reactive antibodies has been included.

Tau structure

Tau gene and isoforms

Human tau protein is encoded by a single MAPT gene located on chromosome 17q21 consisting of 16 exons in total (Neve et al., 1986). In human CNS, six tau isoforms of 37-46 kDa molecular weights are yielded by alternative splicing of 2, 3, and 10 exons from mRNA transcript possessing 11 exons (Table 1). Exons 9-12 of MAPT gene encode four highly conserved imperfect repeats of 30 – 31 amino acids, which are part of the microtubule-binding domain of tau. The six isoforms differ by the presence or absence of two N-terminal inserts of 29 amino acids each generating 0N, 1N or 2N tau isoforms and of the second repeat in C-terminal part encoded by exon 10 to form 3R or 4R isoforms (Fig 2).

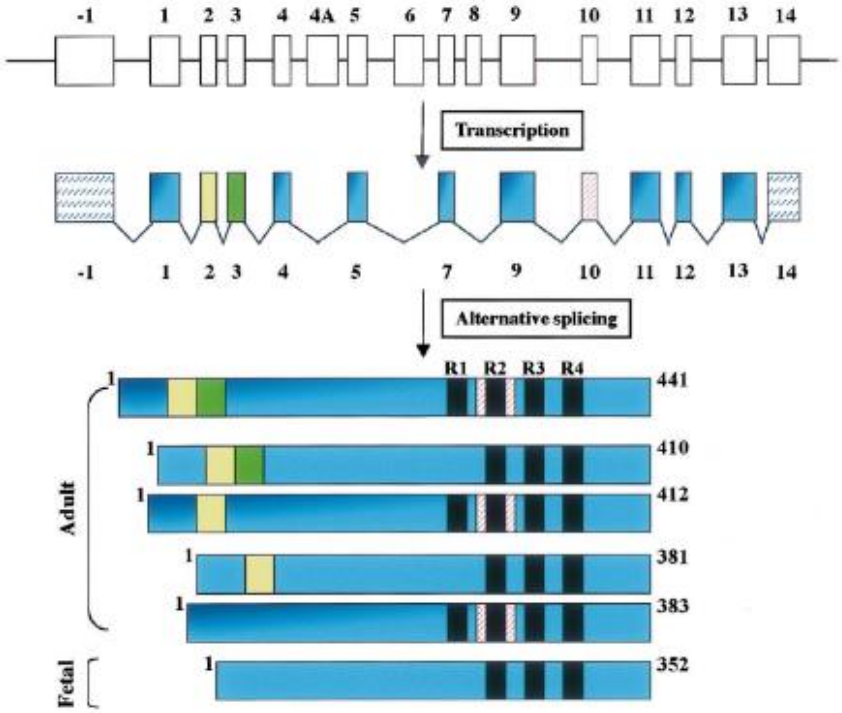


Figure 2: *Schematic representation of the human tau gene, the human tau primary transcript and the six human CNS tau isoforms forming by alternative splicing and occurring in the central nervous system. Adapted from (Buee et al., 2000)*

The distribution of individual tau isoforms varies and significantly depends on brain development stage as well as brain region (Goedert and Jakes, 1990; Takuma et al., 2003, Hanes et al., 2009). Also, differences in tau isoform distribution are described among animal species (Takuma et al., 2003). Several tau isoforms also referred to as a high molecular weight tau or a "big tau" of 110-120 kDa, corresponding to 2N4R tau with moreover exclusively transcribed exons 4a, 6 and 8, are abundant in human PNS (Georgieff et al. 1991; Couchie et al., 1992; Goedert et al., 1992).

Table 1: Isoforms of tau protein and their characteristics in the human central nervous system

Clone	Inserts/Repeats	Alternative exons included	Number of amino acids	Actual MW*	Apparent MW**
htau40	2N4R	2+3+10	441	45.850	67.0
htau39	2N3R	2+3	410	42.967	59.0
htau34	1N4R	2+10	412	40.007	52.0
htau37	1N3R	2	381	42.603	62.0
htau24	0N4R	10	383	39.720	54.0
htau23	0N3R	-	352	36.760	48.0

** Actual MW (molecular weight) is a theoretical molecular weight calculated from amino acids of protein sequence*
*** Apparent MW calculated from retention of tau molecules on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)*

Tau domains and biochemical properties

The full-length tau protein (2N4R, 441 aa) is a molecule of overall hydrophilic (55% amino acids are polar) with the resulting properties such as natively unfolded structure, heat stability and high water solubility. Since tau protein was discovered as an essential factor for microtubule assembly (Weingarten et al., 1975), the two main domains of tau were identified

as a projection domain (Hirokowa et al., 1988) located in N-terminal part and a microtubule assembly domain in C-terminal part by chymotrypsin cleavage at Y197 (Steiner et al., 1990). At present, molecule of tau protein (2N4R) can be divided into four main domains differing by their biochemical properties: N-terminal acidic domain with amino acids 1-150, which contains two N-terminal inserts, proline-rich domain with amino acids 151-243, microtubule binding domain (MTBD) of amino acids 245-369 containing four imperfect repeated regions (~31 aa each) separated by flanking regions, and C-terminal tail of 370-441 amino acids. Tau protein has an overall basic character with an asymmetry in charges distribution influencing its interactions. However, this dipole character of tau molecule can be altered by posttranslational modifications (Kontaxi et al., 2017). The N-terminal part is more acidic (pI ~ 3.8), the following proline-rich domain is of a basic character (pI ~ 11.4) and the C-terminal region is also positively charged (pI ~ 10.8) (Sergeant et al., 2008). The amino acid sequence with polar amino acid labeled and domains of full-length tau are illustrated in (Fig 3A).

Tau protein is a representative of natively unfolded proteins, also termed as "intrinsically unstructured proteins", characterized by a random structure in solution and minimal content of secondary structure (Schweers et al., 1994; Skrabana et al., 2006). As is illustrated in (Fig 3B) adapted from (Mandelkow and Mandelkow, 2012), several amino acid short sequences localized in MTBD (K274-L284, S305-D315, and Q336-D345) transiently populate β-sheet conformation and regions with sequences L114-T123 and L428-A437 transiently assume α-helix formation. In the proline-rich region, three short sequences of amino acids T175-S184, P216-P223, and P232-A239 are prone to form transient polyproline II helical conformation (Mukrasch et al., 2009). All these transient secondary conformation structures significantly participate in tau physiological functions, interactions with binding partners and proper molecule folding as well as pathological tau aggregation (Gamblin, 2005).

Due to the hydrophilic character and very low content of secondary structures, tau protein is a natively unfolded protein with highly flexible conformation in physiological buffers but retains some inherent structure, which can be destroyed by denaturation with 2 M guanidine hydrochloride. The global transient folding of tau protein was revealed as folding back of N- and C-terminal domains over the repeat domain whereby reminds a paperclip-like suprastructure (Jeganathan et al., 2006).

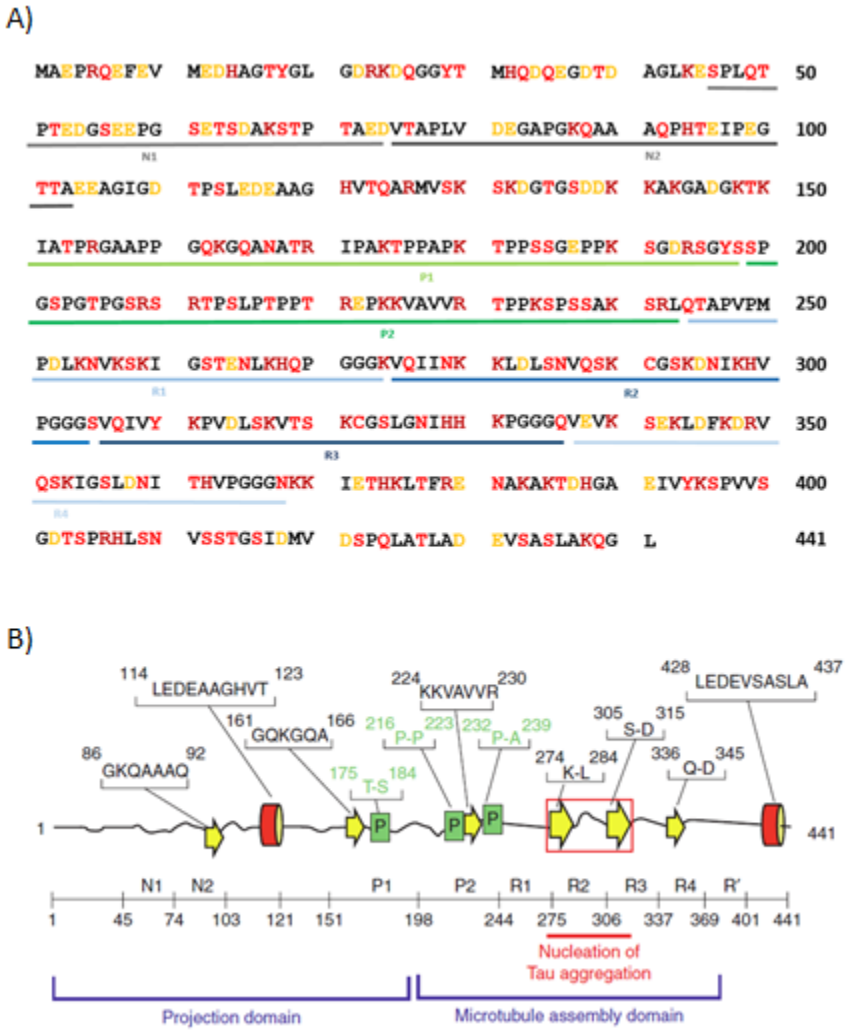


Figure 3: *Tau protein.* ***A)*** *The amino acid sequence of human full-length tau (isoform 2N4R, 1-441 aa). Tau protein contains 243 polar amino acids (basic are dark red, uncharged red, and acidic yellow), and 198 non-polar hydrophobic amino acids (black). Two N-terminal inserts of 29 amino acids (N1 and N2) and imperfect repeated regions (~31 aa each, R1-R4) are underlined.* ***B)*** *Tau domains and structural elements deduced from NMR. The unfolded sequences are represented as black lines intermittent with a few transient elements of secondary structures (red for α-helix, yellow for β-sheets, and green for poly-proline helix), B adapted from (Mandelkow and Mandelkow, 2012)*

Physiological distribution and functions of tau protein

Tau protein is a member of MAPs localized predominantly in axonal projections in neurons of peripheral and central nervous system (PNS and CNS, respectively) (Binder et al., 1985; Peng et al., 1986; Kanai and Hirokawa, 1995). In a lesser extent, tau mRNA and protein were also detected in oligodendrocytes and astrocytes (Couchie et al., 1985; LoPresti et al., 1995; Muller et al., 1997; Klein et al., 2002). Tau subcellular distribution and the expression of different tau isoforms are strictly regulated during the neuronal development. In CNS, tau isoform with three tandem repeats (3R) at the C-terminal part is predominantly represented in the early stages of brain development providing more plasticity in microtubule network formation. During neuronal maturation, the tau isoform with four repeats (4R) begins to occur and dominates in adult neuronal axons (Pizzi et al., 1995; Takuma et al., 2003). The sorting mechanisms of tau into an axonal compartment in mature neurons are not fully understood. But some mechanisms for both mRNA (Litman et al., 1993; Aronov et al., 2001; Malmqvist et al., 2014) and protein level (Hirokawa et al., 1996; Konzack et al., 2007, Scholz and Mandelkow, 2014) have been proposed. A minor part of tau protein is also detected in the somatodendritic compartment (cell body, dendrites, nuclei, and mitochondria) which gradually reveal its new potential physiological functions. Interestingly, this subcellular distribution of tau protein in neurons seems to be isoform-dependent (Liu and Gotz, 2013; Zempel et al., 2017).

Tau protein was discovered as a protein factor essential for microtubule assembly by Kirschner group in 1975 (Weingarten et al., 1975). Tau interacts with microtubules through the repeat domains and flanking regions located at microtubule assembly domain at C-termini (Steiner et al., 1990), as shown in Fig 4. It thereby stabilizes microtubules (Kadavath et al., 2015), promotes their assembly and participates in the reorganization of the cytoskeleton via dynamic interactions with microtubules (Kempf et al., 1996; Elie et al., 2015). Moreover, interactions of microtubules with tau participate in the regulation of axonal transport by several different mechanisms (Trinczek et al., 1999; Dixit et al. 2008).

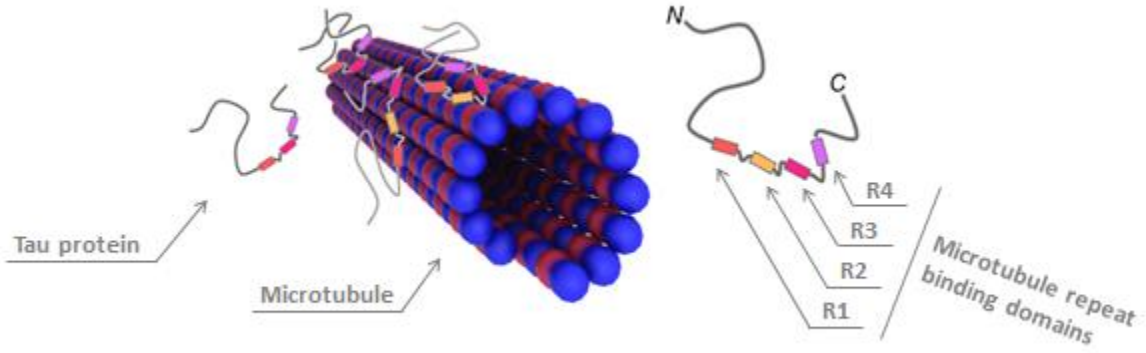


Figure 4: *Tau interactions with a microtubule. Human tau isoforms 0N4R, 1N4R, and 2N4R contain four imperfect repeat domains (R1-R4) that are parts of microtubule assembly domain and participate in interaction with microtubules. Also, tau isoforms 0N3R, 1N3R, and 2N3R that lack R2 domain interacts with microtubules. Adapted from (Choi et al., 2011)*

The N-terminal projection domain of tau protein, which is not involved in interactions with MTs, associates with membranes and may serve as a mediator of microtubule-plasma membrane interactions during the process outgrowth (Brandt et al., 1995). Interestingly, the proline-rich region in N-terminal part of tau binds with membrane-associated signaling molecules containing SH3 domains (for instance src-family tyrosine kinase Fyn) in phosphorylation-dependent manner that shows its potential role in intracellular signaling pathways (Lee et al., 1998; Arrasete et al., 2000; Reynolds et al., 2008).

The evidence of the physiological location of tau protein in dendrites and synapses was proved in several papers, which are summarized in review (Regan et al., 2017). The physiological function of dendritically situated tau has been under intensive investigations. So far, tau protein may play a role in the regulation of morphological plasticity of dendrites (Chen et al., 2012; Pallas-Bazarra et al., 2016). Nuclear tau protein (Loomis et al., 1990; Brady et al., 1995) is implicated in maintenance of genome and chromosomal stability (Rossi et al., 2008) and protection of both nucleic acids, DNA and RNA, respectively (Violet et al., 2014). Recently, it was proven that gene expression may be partly regulated via the interaction of nuclear tau with DNA molecules and via tau participating in ribosomal RNA processing (Frost et al., 2014), but the mechanisms are still poorly understood.

The physiological localization of tau protein is essential for its proper functions that are briefly summarized above. Under pathological conditions, missorting of tau protein accompanying by pathological post-translational modifications and aggregation leads to losing its normal functions and to neurodegenerative processes inside the stricken neurons. Thus, the next chapter is devoted to post-translational modifications of tau protein, which play a crucial role in both physiological tau properties as well as disease-associated changes.

Post-translational modifications of tau protein

Co-/post-translational modifications (PTMs) are a biological mechanism by which the functional diversity of proteins is expanded. Especially, intrinsically disordered proteins (IDPs) demonstrate vulnerability to be abundantly post-translationally modified and thereby may significantly change their structural properties affecting binding promptness and functions (Bah and Forman-Kay, 2016). Tau protein as a typical representative of IDPs is highly regulated by a variety of PTMs. Phosphorylation, truncation and conformational changes leading to tau oligomerization and aggregation are most studied PTMs of tau protein in relation to form tau pathological forms observed in brain tissue of AD patients and are discussed in more details later in this chapter. Otherwise, additional numerous PTMs of tau protein are also known and their role in tau function has been intensively studied (Morris et al., 2015) and excellently reviewed in the literature (Pevalova et al., 2006; Martin et al., 2011; Kontaxi et al., 2017). Even though each PTM is formed by a distinct mechanism utilizing different enzymes, cofactors and chemical groups to modify amino acid residues, their overlapping has a great impact on tau function, its cellular localization, and turnover (Venne et al., 2004; Gong et al. 2005; Yang and Seto, 2008). Multiple lysine residues occurring in tau protein molecule (44 lysine residues in human full-length tau variant 2N4R) may be modified with lysine-directed PTMs (glycation, acetylation, ubiquitination, sumoylation, and methylation) which play role in tau assembly and toxicity via participating in electrostatic and hydrophobic interactions (Kontaxi et al., 2017). N-glycosylation, O-GlcNAcylation, prolyl-isomerization, nitration, polyamination, and oxidation are other PTMs discussed in relation to the tau-mediated pathogenesis of AD.

Tau phosphorylation

Tau is a multiply phosphorylated protein as was demonstrated at the turn of 70th and 80th of the last century in relation to its ability to modulate cytoskeleton dynamics (Cleveland et al. 1977; Selden and Pollard, 1983; Lindwall and Cole, 1984). As tau properties in both physiological/pathological circumstances have been gradually discovered, phosphorylation has become the most studied PTM. As in case of tau isoform expression (Goedert et al., 1989), site-specific phosphorylation of tau protein is developmentally regulated. Fetal tau protein is phosphorylated in higher rates than tau in mature neurons (Brion et al., 1993; Yu et al., 2009). Although only 0N3R tau variant is found in the fetal brain, its increased phosphorylation has a partial contribution to the weakened affinity of tau to MTs, in this case reflecting the period of active neurite outgrowth and neuronal polarization (Yu et al., 2009). In mature neurons, tau PTMs, including phosphorylation, are likewise dynamically processed and participate in proper functions and physiological maintenance of neurons. Tau phosphorylation state may influence also additional features connected with MT dynamics and stability, such as axonal transport and synaptic plasticity (Tatebayashi et al., 2004; Mondragón-Rodríguez et al., 2012). In general, tau phosphorylation has a great impact on tau interactions with binding partners, its localization, and function.

In AD and other related tauopathies, the occurrence of altered tau protein hyperphosphorylated in a site-specific manner results in decreased MT binding leading to tau missorting and aggregation into neurofibrillary deposits (review: Wang et al., 2013). Tau protein is a natively unfolded protein and contains 85 phosphorylatable residues (45 serines, 35 threonines, and 5 tyrosines calculated for human full-length tau 1-441 aa), thus is considered as an ideal substrate for multiple kinases and phosphatases. The imbalance in tau kinase and phosphatase activities may trigger the non-physiological tau phosphorylation with all the consequences leading to neurodegeneration (Billingsley and Kincaid, 1997; Gong et al., 2006). The fact that tau protein is highly phosphorylated in AD brains versus control brains has been documented. Normal brain tau has 2-3 mole of phosphate per mole of protein, but AD brains also contain tau with an approximately three-fold greater stoichiometry of mole of phosphate/mole of tau (Kopke et al., 1993). So far, 45 phosphorylation sites were detected in insoluble aggregates of tau extracted from AD brain, herein referred to PHF-tau (Fig 5), several of them are strictly

AD-specific and some of them are shared with tau isolated from control brains (Martin et al., 2013; Noble et al., 2013). The phosphorylation of tau protein is a highly dynamic process, thus, even though multiple phosphorylation sites were detected in tau from both in control and AD brains (Martin et al., 2013), tau phosphorylation order and alterations have been still under continual investigation. Moreover, in some phosphorylation clusters (e.g. S210-T217, T231-S238), there was proven hierarchical phosphorylation, meaning that phosphorylation occurs sequentially with specific initial phosphorylation sites which prime for further phosphorylation in nearby residues (Goedert et al., 1994; Hanger et al., 2007). The phosphorylation sites in PHF-tau are predominantly located in the proline-rich domain and the regions flanking the microtubule-binding domain (Morishima-Kawashima et al., 1995; Hanger et al., 1998; Vega et al., 2005; Hanger et al., 2007, Noble et al., 2013) and are involved in alterations in tau-microtubule binding dynamics and interactions with other reactive partners. So far, four phosphorylated residues occurring specifically in PHF-tau have been identified in MBD region (S258, S262, S289, and S356) and were shown to have a substantial impact on the reduction of tau binding to microtubules (Biernat et al., 1993; Ando et al., 2016).

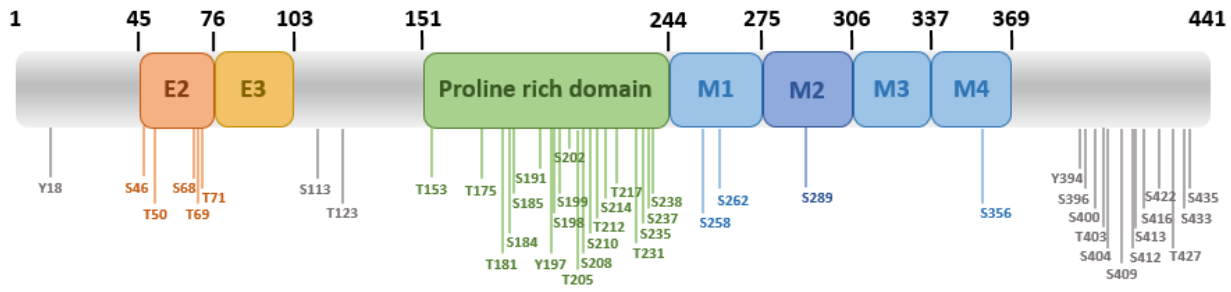


Figure 5: *Phosphorylation sites (45) detected in insoluble aggregates of tau extracted from AD brain referred as PHF-tau, with color-coded domains.*

A large number of kinases and phosphatases are able to phosphorylate tau as was shown in many *in vitro* experiments. The kinases may be divided into three main groups; first, two belongs to Ser/Thr kinases: 1) proline-directed protein kinases (PDPKs), 2) non-proline-directed protein

kinase (NPDPKs), and 3) tyrosine kinases (TKs). But it is necessary to bear in mind that not all the kinases phosphorylating tau *in vitro* have yet been proven to phosphorylate tau *in vivo* and may only indirectly regulate tau phosphorylation (Johnson and Stoothoff, 2004). Individual kinases and phosphatases, which are considered to be involved in (de)phosphorylation of tau and may contribute to AD pathology, are described in more details below.

Proline-directed protein kinases

There are at least three different PDPKs families involved in tau-based AD pathology: GSK3, MAP kinases, and Cdks showing slightly different substrates´ preferences.

Glycogen synthase kinase-3 (GSK-3)

GSK3 is ubiquitously expressed mammalian proline-directed Ser/Thr kinase, which is a key regulator of multiple signaling pathways phosphorylating numerous protein substrates (reviewed by Frame and Cohen 2001; Woodgett, 2001). Two isoforms of GSK-3 were identified, GSK-3α and GSK-3β (Woodgett, 1990). There are two peculiarities linked to GSK-3 in comparison to other kinases. Firstly, GSK-3 is constitutively active (phosphorylated at T216) in most tissues and its activity is mostly regulated by inhibitory phosphorylation at S9 (Harwood, 2001). Secondly, GSK-3β is more prone to phosphorylate primed substrates (pre-phosphorylated on particular Ser/Thr residues) with sequences S/T-X-X-X-pS/pT (Fiol et al., 1987). This priming of the substrate is not strictly required but greatly increases the efficiency of GSK-3-mediated substrate phosphorylation. Tau protein is a suitable GSK-3 substrate in both primed and unprimed state, though they differ in microtubule binding after GSK-3-mediated phosphorylation (Sengupta et al., 1998; Cho and Johnson, 2003). Thus, tau phosphorylation by multiple kinases may diversely affect tau properties and influence its roles in physiology and AD pathology. Among kinases involved in tau pathology, GSK3 is associated with the largest amount of putative phosphorylation sites at tau molecule. Approximately 40 residues were identified to be phosphorylated by GSK-3 *in vitro*; 13 of them were exclusively found in PHF-tau (T69, T153, T175, S184, S185, S210, S214, S237, S258, S262, S289, S356, and S409, respectively) (reviewed by Hanger and Noble, 2011). Levels of active form (GSK-3-pT216) are increased in AD brain where accumulate with tau deposits in neurons (Leroy et al., 2007). As GSK-3 regulates many cellular signaling pathways, it is regarded that active GSK-3 has an impact

on more aspects of AD development and progression (reviewed by Llorens-Maritin et al., 2014), which even led to establishing the GSK3 hypothesis of AD (Hooper et al., 2008). Much discussed topics are GSK-3 as a mechanical link between two major pathological hallmarks, tau deposits and Aβ toxicity (Terwel et al., 2008; Dunning et al., 2015) and GSK-3 as a promising therapeutic target in AD treatment (reviewed by Maqbool et al., 2016).

Mitogen-activated protein kinases (MAPKs)

Another proline-directed S/T kinase family investigated in relation to tau-associated AD pathology are three MAPK groups: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and p38 (Atzori et al., 2001; Ferrer et al., 2001). All these MAPK subfamilies contain several isoforms. Two ERKs isoforms are known as ERK1 (p44-MAPK, MAPK3) and ERK2 (p42-MAPK, MAPK1), respectively (Boulton et al., 1991; Childs and Mak, 1993). JNKs, also known as stress-activated protein kinase or SAPK, exist in several alternatively spliced isoforms JNK1-3 (Gupta et al., 1996), and p38 isoforms α, β, γ, and δ have been identified so far (Cuenda and Rousseau, 2007). Multiple MAPK pathways, activated by both extracellular and intracellular stimuli, regulate diverse key cellular activities (cell proliferation, differentiation, apoptosis, synaptic plasticity etc.) via mediated phosphorylation of their substrates (reviewed by Roux and Blenis, 2004; Thomas and Huganir, 2004). Tau protein is phosphorylated by MAPK in physiological manners as well as under pathological circumstances (Drewes et al., 1992). In AD brains, all three MAPK subfamilies (ERK, JNK, and p38) have been demonstrated to be differentially expressed in association with tau deposits in neurons. Moreover, active forms of MAPKs are increased in AD compared to controls (Trojanowski et al., 1993; Hensley et al., 1999; Atzori et al., 2001; Ferrer et al., 2001; Pei et al., 2001; Zhu et al., 2001). Eleven tau S/T residues found in PHF-tau were identified to be phosphorylated by all three MAPK subfamilies: T175, T181, S202, T205, T212, T217, S231, T235, S396, S404, and S422. Individually, ERK phosphorylates tau at S46, T50, T69 and 153, and p38 at T245, S305, and S356, respectively (Reynolds et al., 2000; Martin et al., 2013; Wang et al., 2013). All these published data indicate that MAPKs are kinases playing their role in tau pathology under stress conditions and may be possible targets for therapeutic interventions (Harper and Wilkie, 2003).

Cyclin-dependent kinase 5 (Cdk5)

Cdk5, a member of cyclin-dependent kinases (Cdk), is a proline-directed Ser/Thr kinase. In contrast to other Cdks actively operating in proliferating cells, Cdk5 is predominantly activated in post-mitotic neurons via binding with its neuron-specific activators p35 or p39 (Lew et al., 1994). Since soluble Cdk5 is highly expressed in neurons, the kinase activity is mainly determined by accessible levels of activators, which are rapidly degraded by ubiquitin-proteasome pathway (Minegishi et al., 2010). Under stress conditions, the calpain-mediated truncation of membrane-associated p35 subunit releases the C-terminal fragment p25 with a longer half-life that is translocated from membranes and causes overactivation of Cdk5 (Patrick et al., 1999; Lee et al., 2000). This hyperactivated complex Cdk5-p25 is thought to be involved in abnormal tau phosphorylation in AD (Imahori and Uchida, 1997; Cruz and Tsai, 2004). Some of tau S/T residues were approved to be phosphorylated by Cdk5 *in vitro*, namely S202, T205, S235 and S404 (Imahori and Uchida, 1997), all detected in both PHF-tau and physiological tau (Martin et al., 2013). Besides major *in vitro* Cdk5 sites of tau, also several more tau phosphorylation sites are listed in other publications (Paudel et al., 1993; Liu et al., 2002). To date, the studies focusing on determination of activator p25 levels in AD post-mortem brains and the effect of Cdk5-p25 complex on NFT formation in transgenic animal models have given controversial results (critically reviewed at Giese et al., 2005; Giese, 2014). The complex Cdk5-p25/p35 also phosphorylates proteins involved in synaptic plasticity and memory formation, plays a role in neuronal differentiation and cross-talks with GSK-3β (Tsai et al., 1993; Fischer et al., 2005; Engmann and Giese, 2009). These are only several examples of pathways, which Cdk5 orchestrates, and it is necessary to better understand the role of Cdk5 and its co-activators under both physiological and pathological circumstances.

Non-proline-directed protein kinases

cAMP-dependent protein kinase

cAMP-dependent protein kinase (PKA) was the first protein kinase which structure was determined and since that over 370 substrates have been identified. PKA belongs to non-proline directed S/T protein kinases within the AGC kinase subfamily. The inactive PKA heterotetrameric R2C2 complex is composed of two catalytic (C) and two regulatory (R)

subunits. In PKA signaling pathways, the binding of second messenger 3',5'-cyclic adenosine monophosphate (cAMP) to the R subunits is responsible for C subunits release and a subsequent substrate phosphorylation (Taylor et al., 1990; Shabb, 2001; Pearce et al., 2010). The ubiquitous distribution of PKA and its diverse cellular localization mediated via multidomain scaffolding proteins result in a large number of potential PKA substrates affecting physiological functions. Tau protein has been intensively studied as a physiological PKA substrate since about a third of tau Ser/Thr sites were identified as a target for *in vitro* PKA-mediated phosphorylation. Nine of them are supposed to be strictly AD-specific phosphorylation sites: S208, S210, S214, S258, S262, S356, S409, S422, and S435 (Jicha et al., 1999; Hanger et al., 2007; reviews: Sergeant et al., 2008; Hanger et al., 2009; Martin et al., 2013; Wang et al., 2013). PKA was also confirmed as an effective priming kinase for GSK-3 residues specificity, which facilitates tau further phosphorylation by GSK-3 at multiple sites (Liu et al., 2004; Liu et al., 2006). The mechanism of priming tau protein has not been yet fully understood, but in contrary, two from ten putative GSK-3 phosphorylation sites in PKA-prephosphorylated tau are inhibited, also there is the totally distinct effect of PKA prephosphorylation on Cdk5-mediated tau phosphorylation (Liu et al., 2006). The cooperation of various kinases and priming mechanisms may lead to different site-specific preferences of kinases, which subsequently phosphorylate primed tau protein.

Casein kinase 1 superfamily

Casein kinase 1 superfamily contains casein kinase 1 (CK1, isoforms: α1, α2, γ1, γ2, γ3, δ, and ε), tau-tubulin kinase (TTBK, isoforms 1 and 2), and vaccinia-related kinase (VRK, isoforms 1, 2 and 3) (Manning et al., 2002; Ikezu and Ikezu, 2014). Within this kinase superfamily, CK1δ and both TTBK1/2 were proven to be involved in tau phosphorylation in PHF-tau manner. CK1δ is S/T specific kinase that phosphorylates cytoskeletal structures like α/β tubulin and MAPs, including tau protein (Behrend et al., 2000). *In vitro* study, Ck1δ-mediated tau phosphorylation generates several PHF-associated sites and disrupts microtubule binding (Li et al., 2004). The elevated levels of CK1δ protein and mRNA, respectively, are found in affected areas of AD brains (Ghoshal et al., 1999; Yasojima et al., 2000) and are associated with pathological tau accumulation in brains of AD and other several tauopathies (Schwab et al., 2000). TTBK1 is neuronal specific S/T/Y protein kinase which expression is upregulated in AD brains (Sato et al., 2008) and co-localized with pre-tangle S422-

phosphorylated tau formations (Lund et al., 2013). Tau protein is a substrate for both TTBK1 and 2 isoforms phosphorylated tau at PHF-specific sites *in vitro*; Tyr197, S198, S199, S202, and S422 by TTBK1 (Sato et al. 2006), and S208 and S210 by TTBK2, respectively (Tomizawa et al., 2001).

Dual-specificity tyrosine-regulated kinases

Mammalian dual-specificity tyrosine-regulated kinases (DYRKs) subfamily consists of 5 members Dyrk1A, Dyrk1B, Dyrk2-4 with genes located on different chromosomes (Becker et al., 1998). The dual specificity is characterized by their ability to auto-phosphorylate tyrosine in the catalytic loop for self-activation and to phosphorylate target proteins on S/T residues. They play regulatory roles in signaling of cell proliferation, survival, and development. The most studied Dyrk linked to neurodegeneration is Dyrk1A because of its gene location on chromosome 21 (Kentrup et al., 1996) which is associated with neurodevelopmental alterations in Down syndrome (review: Park et al., 2009) and involvement in brain development (Dierssen et al., 2006; Guedj et al., 2012). In AD, Dyrk1A mRNA level is elevated (Kimura et al., 2006), and the alterations in expression of constitutive Dyrk1A in the cytoplasm and nuclei of neurons also occur (Ferrer et al., 2005). Dyrk1A participates on tau phosphorylation at position Thr212 *in vitro*, which primes tau to be phosphorylated by GSK-3β at S208 (Woods et al., 2001), a position identified as a strictly AD-specific phosphorylation site (Martin et al., 2013).

Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), microtubule-affinity regulating kinases (MARKs), phosphorylase kinase (PhK), protein kinases PKN and PKC, and protein kinase B (PKB/Akt) are other non-PDPKs associated with tau pathology. They can directly phosphorylate non-S/T-P motifs mostly located in MTBDs of tau or indirectly affect other kinase activities (Correas et al., 1992; Singh et al., 1996; Paudel, 1997; Kawamata et al., 1998; Sironi et al., 1998; Ksiezak-Reding et al., 2003; Matenia and Mandelkow, 2009).

Tyrosine kinases

Most published studies about phosphorylation involved in tau-based AD pathological changes are dedicated to serine/threonine residues in tau protein and the role of tau tyrosine phosphorylation and searching for potential tyrosine kinases in AD pathogenesis have only recently emerged. All six tau isoforms located in human CNS possess five tyrosine residues: Y18, Y29, Y197, Y310, and Y394 (numbered according to the longest human tau isoform 2N4R, 1-441 aa). The increasing evidence of tyrosine kinases involvement in AD has been documented in relation to their altered levels, missorting localization, and occurrence within tau deposits in AD brains compared to control subjects (*Shirazi and Wood, 1993; Ho et al., 2005*). Moreover, particular tyrosine phosphorylated tau was proved to be a part of PHF-tau and tau deposits (Lee et al., 2004; Vega et al., 2005). Src family, TTBK1, and c-Abl are the most intensively discussed tyrosine kinases contributing to tau phosphorylation.

Src family of non-receptor tyrosine kinases (SFKs) containing SH3 domains are ubiquitously expressed. Interestingly, tau is a suitable substrate candidate for interactions with SFKs since possessing seven potential SH3 binding motifs (PxxP) at the proline-rich region in N-terminus (Lee et al., 1998). The family members Src, Fyn, Lck, and Lyn are found in brain neurons (Cooke and Perlmutter, 1989; Umemori et al., 1992; Omri et al., 1996; Yanagi et al., 2001) and linked with phosphorylation of tyrosine residues at tau (Lee et al., 1998). The major tau tyrosine residue phosphorylated by SFKs is Y18 (Lee et al., 2004; Lebouvier et al., 2008). This phosphorylation residue seems not to alter interactions of tau with MTs but is a part of PHF-tau and occurs in NFT-bearing neurons (Lee et al., 2004). Later, a study published MS analysis of *in vitro* tau phosphorylation detected more phosphorylated tau tyrosine residues by Src family members: Y18, Y197, Y310, and Y394 in reaction with Lck, and Y18 and Y197 phosphorylated with Fyn (Scales et al., 2011).

Tau phosphorylated at Y197 was detected in AD brains (Vega et al., 2005). Sato and colleagues proved that tau-tubulin kinase 1 (TTBK1) in addition to several S/T residues occurred in PHF-tau also directly phosphorylates tau at Y197 residue *in vitro* (Sato et al., 2006). TTBK1 is

a neuron-specific dual kinase, which phosphorylates S/T/Y and belongs to casein kinase 1 superfamily as was mentioned previously in this chapter.

Phosphorylation of tau at position Y394 has been also investigated since Y394-phosphorylated tau was found both in PHF-tau isolated from AD brains and in human fetal brain tau (Derkinderen et al., 2005). The kinases which were so far shown to phosphorylate tau at Y394 are c-Abl and Arg (Abl-related gene, also known as Abl2) (Tremblay et al., 2010); both belong to Abl family of mammalian non-receptor tyrosine kinases and are implicated in cellular responses, mostly apoptosis, oxidative and other types of stress (Sun et al., 2000; Cao et al., 2001).

Taken together, phosphorylation of tyrosine residues in tau protein may have a role in AD progression and more investigation on this topic is certainly needed.

Phosphatases

Abnormal phosphorylation of tau protein in site-specific manners is associated with loss of tau biological function accompanied by aggregation into PHFs. Since these filamentous structures composed mostly of hyperphosphorylated tau form various intracellular deposits with gain the toxic function, the balance between tau kinases and phosphatases activities is supposed to be a crucial mechanism, by which the state of tau phosphorylation is controlled. Besides kinases participating in abnormal tau phosphorylation, several phosphatases involved in tau biology have been identified and the failure in their physiological activity is suggested. Protein phosphatases types 1, 2A, 2B (known also as calcineurin), and 5 (PP1, PP2A, PP2B, and PP5, respectively) are the major protein phosphatases responsible for regulation of tau phosphorylation state in brain (Yamamoto et al., 1988; Drewes et al., 1993; Gong et al., 1994 a-c; Liu et al., 2005a, b; and excellently reviewed by Liu et al., 2006). Phosphatases have generally wider substrate-specificities in comparison to most kinases, thus each phosphatase dephosphorylates numerous phosphorylated S/T residues in PHF-tau but with various efficiencies toward different sites (Liu et al. 2006).

Importantly, in 2005, Liu and coworkers published an outstanding original article regarding the contribution of individual phosphatases on the regulation of tau phosphorylation. The results show that PP1, PP2B, and PP5 accounted together for approximately 30% of the total tau phosphatase activity of human brain (11% for PP1, 7% for PP2B, and 10% for PP5, respectively) and the remaining 71% of total tau phosphatase activity is provided by PP2A (Liu et al., 2005b). This conclusion is in agreement with data that PP2A is the major phosphatase that regulates tau phosphorylation to the extent that MT assembly promoting the activity of tau is significantly decreased by specific inhibition of PP2A (Sontag et al., 1996; Gong et al., 2000). The activity of all phosphatases involved in tau dephosphorylation were shown to be compromised in AD (Gong et al. 1993, Sontag et al. 2004, Liu et al. 2005 a-c), but it is evident that the partial contribution of individual phosphatases has to be included to fully understand the overall effect of each phosphatase on regulation of tau phosphorylation state in AD pathology. In summary, abnormal tau phosphorylation in AD is not only a result of altered kinase activities, but the impairment in regulation and activity of phosphatases may also significantly contributed to the formation of PHF-tau and their impact on tau phosphorylation state should not be underestimated.

Tau truncation

Various structure types of aggregated tau forming intracellular deposits is a histopathological hallmark of neurodegenerative disorders named tauopathies, including AD. Besides phosphorylation, tau truncation is another important PTM that participates along with conformational changes in initiation and/or acceleration of tangle formation (Binder et al., 2005; de Calignon et al., 2012; Mead et al., 2016). Tau protein is a natively unfolded protein and therefore sensitive to protease cleavage both *in vitro* and *in vivo*, summarized in reviews (Wang et al., 2010; Chesser et al., 2013). To date, endogenous proteases involved in tau fragmentation and precise cleavage sites have been only partially known. But better understanding of the formation and action of tau truncated species is needed for several reasons; (1) active forms of several endogenous proteases implicated in tau cleavage are elevated in AD brains (Rohn et al., 2002; Ramcharitar et al., 2013), (2) tau fragments might be intrinsically toxic to neurons *per se* and/or possess enhanced tau pro-aggregation properties (Zilka et al., 2006; de Calignon et

al., 2012); (3) occurrence of several fragmented tau species correlates with AD progression (Basurto-Islas et al., 2008), and (4) amyloid and tau pathology may be mechanically linked to activity of several proteases (Cotman et al. 2005; Park and Ferreira, 2005).

To date, caspases and calpains have been the most intensively studied endogenous proteases in relation to tau fragmentation leading to tangle formation. Caspases belong to a family of cysteine-dependent aspartate-directed proteases and their activation is a key biochemical event in a programmed cell death pathway termed apoptosis (Lazebnik et al., 1994; Alnemri et al., 1996). Though caspases are proteases mainly associated with apoptosis, there is evidence of non-apoptotic caspases´ roles, which may lead to cleavage of many cellular proteins and contribute to neuronal pathology prior to cell death (Hyman, 2011; Hyman and Yuan, 2012). Moreover, elevated caspase activity in AD brains and its correlation with AD pathology were proven (Rohn et al., 2001; Su et al., 2001; Guo et al., 2004). Tau protein was identified as one of many caspase substrates (Canu et al. 1998; Fasulo et al., 2000; Gamblin et al., 2003). The only caspase-mediated tau cleavage site validated both *in vitro* and *in vivo* is at D421 position (D421-S422 calculated for tau 1-441 aa) mediated by multiple caspases, most likely by caspase-3 (Gamblin et al., 2003; Rissman et al., 2004). This C-terminally truncated form of tau, also called TauC3, is implicated in tau aggregation and seeding activity linked to cell toxicity (Nicholls et al., 2017). Other putative cleavage sites for caspases may occur in both C-terminal and N-terminal parts, for instance, Asp13 at its N-termini cleaved by caspase 6 (Horowitz et al., 2004). Active forms of calpains (forms calpains-1 and -2), calcium-activated neutral proteinases, were also found to be increased in AD-affected brains (Saito et al., 1993). This phenomenon might be explained as a consequence of disturbances in calcium influx and decreased levels of calpastatin, a calpain endogenous inhibitor, both events described in AD (Rao et al., 2008; Magi et al., 2016). Calpain-mediated tau cleavage generates 17kDa tau fragment with intrinsic toxic effect (Park and Ferreira, 2005; Reinecke et al., 2011). Ferreira´s group detected its occurrence only in AD- and other tauopathies-affected brains compared to controls (Ferreira and Bigio, 2011). In these works, the amino acid sequence of 17kDa fragment was determined as residues 45-230 due to the predicted calpain-cleavage sites (Canu et al., 1998). In Mandelkow´s laboratory, the amino acid sequence of calpain-mediated 17kDa cleavage tau product was determined to comprise residues 125–230 (Garg et al., 2011). And, in contrary, they detected this fragment even in control brains and did not confirm its cytotoxic effect in the cell-based system (Grag et al., 2011). The Aβ-

induced generation of 17kDa tau fragment via calpain activation was proven in both laboratories, but its role in AD pathology has not been yet fully evaluated.

Thrombin, a serine protease, and its precursor prothrombin are also localized in brain tissue. Thrombin was shown to be elevated in the brain and cerebral microvasculature in AD, and also thrombin-positive NFTs were detected by immunohistochemistry (Arai et al., 2006; Yin et al., 2010). Thrombin was proven to degrade tau protein *in vitro* from its N-terminus by proteolysis on multiple arginine and lysine sites, including R155-G156, R209-S10, R230-T231, K257-S258, and K340-S341 (Arai et al., 2005). The first thrombin cleavage site was identified at R155-G156 bond yielding a 25-kDa C-terminal fragment (Olesen, 1994) which is continuously degraded (Arai et al. 2005).

In AD, several other tau fragments have been identified but the process of their formation has not yet been clearly established. One of the intensively studied truncations of tau protein is at position Glu391 since such a fragmented tau has been found to be a part of PHFs forming NFTs (Harrington et al., 1991). Glu391-cleaved tau fragment was detected in both early deposits of abnormal tau in cells vulnerable to NFTs degeneration in AD brains (Mena et al., 1991; Mena et al., 1996; Abraha et al., 2000) as well as in NFTs present in certain brain areas at late stages of AD where even corresponds with the severity of tau pathology (Garcia-Sierra et al., 2001; Garcia-Sierra et al., 2003; Basurto-Islas et al., 2008). The *in vitro* polymerization experiments with glu391-truncated tau revealed increased rates of polymerization stimulated by inductors such as arachidonic acid in comparison to full-length tau (Abraha et al., 2000). Even this site-specific truncation of tau protein is associated with tau pathology propagation, endogenous proteases responsible for cleavage of tau at position E391 has not yet been identified. Novak´s group identified truncated tau species with a conformational state different from normal healthy tau which may play a pivotal role in AD neurofibrillary degeneration. These structurally different tau species, truncated at amino acid positions 151-391/421 and specifically reactive with a conformational DC11 antibody, may promote the neurofibrillary pathology of AD type *in vivo* (Vechterova et al., 2003; Zilka et al., 2006).

Tau protein, which is primarily located in cytosol, is degraded and eliminated by proteasomal system (David et al., 2002; Grune et al., 2010) and autophagy machinery (Wang et al., 2009), but both these degrading pathways are prone to be altered under pathological

circumstances such as found in AD (Keller et al., 2000; Nixon and Yang, 2011; Wang and Mandelkow, 2012; Lee et al., 2013). Puromycin-sensitive aminopeptidase (PSA), an aminopeptidase cleaving their substrates from the N-termini, and asparaginyl endopeptidase (AEF), which cleaves protein substrates on the C-terminal side of asparagine, are described as other potential proteases implicated in tau fragmentation. PSA is an alanyl peptidase with high activity in brain tissue (McLellan et al., 1988) and is able to proteolyze tau *in vitro* (Karsten et al. 2006; Sengupta et al., 2006). On contrary, other studies do not confirm tau protein as a direct substrate for PSA, but they suggest PSA as an indirect modulator of tau levels (Chow et al., 2010; Kudo et al., 2011) more likely by promoting tau clearance and involving in autophagy pathway (Chesser et al., 2013). Levels of activated AEP, lysosomal cysteine proteinase, are significantly increased in AD brains and even its translocation from neuronal lysosomes to the cytoplasm is enhanced in AD neurons (Basurto-Islas et al., 2013; Zhang et al., 2014). AEF cleaves tau protein at N255 and N368 residues. Tau with the second cleavage site increased aggregation rate and was shown to be elevated in AD brains and to have a cytotoxic effect on neurons (Zhang et al., 2014). Another group of lysosomal proteases, cathepsins, are also associated with tau fragmentation but more data are needed to better understand their role in tau aggregation and clearance (Bednarski and Lynch, 1996; Kenessey et al., 1997; Wang et al., 2009).

The coordination between tau proteolytic processing and clearance of tau by degrading pathways is essential for maintaining appropriate tau intracellular levels and its functional properties (Chesser et al., 2013). Fragmentation, polymerization, and clearance of tau are interconnected processes and their balance is inevitable for neuronal survival, as well as its disruption, may trigger detrimental events leading to neurodegeneration.

Tau aggregation and paired helical filaments

The fibrils observed in neurons of cerebral cortex in AD were termed paired helical filaments (PHFs) in 1963 (Kidd, 1963) and were characterized as a left-handed double helices of subunits resembling ribbon-like structures with a periodicity of ~ 65–70 nm and thicknesses of ~ 9–18 nm (Wischik et al., 1985; Wegmann et al., 2010). Tau protein was identified as their main component (Kosik et al., 1986; Ihara et al., 1986). Since that, chemical and physical

properties of tau molecule that contribute to aggregation have been gradually revealed. Tau protein is natively unfolded protein of rather a hydrophilic character lacking stable secondary structure/rigid globular domain in solution (Skrabana et al., 2006). The fact that tau is a representative of proteins with intrinsically disordered character does not necessarily mean that tau is a total random coil. Tau rather retains a global hairpin folding structure in solution (Jeganathan et al., 2006) but still preserves substantial flexibility prone to self-assembling and disorder/order transitions depending mostly on ambient conditions, PTMs and specific interactions. The critical point when physiologically active tau molecule becomes a part of pathological PHFs has not yet been fully understood and seems to depend on numerous factors. Studies about PHF formation and tau aggregation could be divided into two main approaches; (1) presence of certain sequence motifs in tau possessing enhanced propensity for β-structures as elements responsible for the abnormal tau aggregation to PHFs called "pro-aggregation motifs", (2) PTMs, mostly abnormal truncation and phosphorylation, contributing to changes in conformational state of tau leading to detaching from MTs, misfolding and self-aggregation. Both approaches complement each other.

The minimal protease resistant tau unit, named PHF core, responsible for tau fibrillization was identified as a 93–95 amino acid residues long tau fragment within the MT-binding domain of sequence beginning in the vicinity of H268 and ending E391at C-terminus (Wischik et al., 1988; Jakes et al., 1991; Novak et al., 1993). Thus, specific pro-aggregation motifs most studied in the context of PHF formation are located within the MT-binding domain at tau C-termini (Okuyama et al., 2008; Sugino et al., 2009). Two candidate hexapeptides clustering the strong β-sheet inducing residues V, I, Y, and Q are of sequences 275-VQIINK-280 (PHF6*) and 306-VQIVYK-311 (PHF6) and localize at the beginning of R2 and R3 domains, they were defined as minimal interaction motifs with predicted β-conformation responsible for assembly of tau into PHFs (von Bergen et al., 2000), shown in Fig 6. Mainly, PHF6 peptide seems to be responsible for the nucleation phase in fibrillization and the positively charged K311 may be essential amino acid in this process (Li and Lee, 2006).

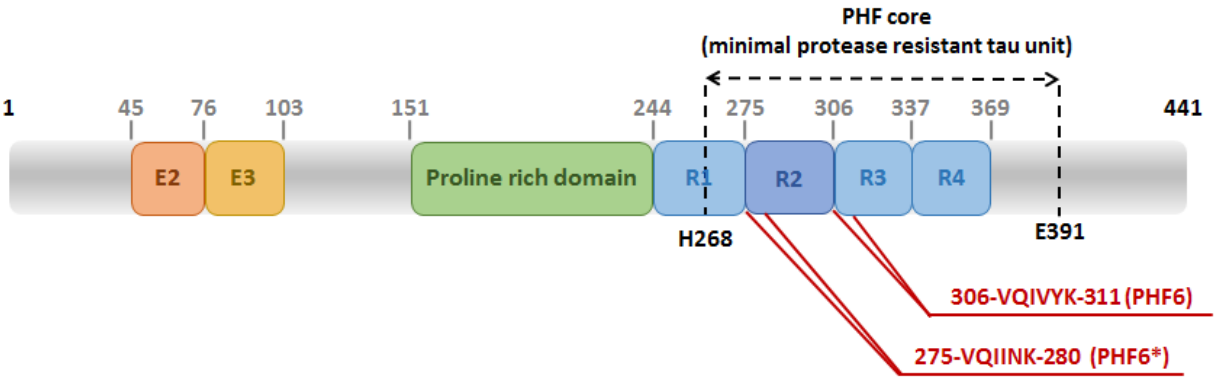


Figure 6: *Full-length tau (2N4R, 1-44 aa) with highlighted minimal protease resistant tau unit, named paired helical filament (PHF) core, responsible for tau fibrillization. Especially, two motifs predicted to possess β-sheet conformation within this PHF core has pro-aggregation properties and are supposed to be the minimal interaction motifs during PHF formation, 306-VQIVYK-311 (PHF6 motif) and 275-VQIINK (PHF6* motif).*

With the onset of anti-tau antibodies specifically recognizing newly exposed and discontinuous epitopes, truncated forms and sequences with pathological phosphorylation residues, the evolution of PHFs started to be investigated also from the perspective of conformational changes and PTMs (summarized in: Binder et al., 2005; Mondragon-Rodriguez et al., 2008; Bibow et al., 2011). It is suggested that tau protein has to undergo numerous conformational changes at the beginning of aggregation and that PHFs are dynamic structures subjected to maturation changes associated with truncation and phosphorylation during the polymerization process. The aim of such concepts is explanation of the ordered series of events in NFTs evolution observed by immunohistochemical studies (Augustinack et al., 2002; Garcia-Sierra et al., 2003; Binder et al., 2005) and supported also by *in vitro* tau polymerization assays (Abraha et al., 2000; Berry et al., 2003). Under physiological conditions, tau protein is bound to MTs and/or takes very flexible suprastructure resembling global hairpin folding in soluble form (Jeganathan et al., 2006). From the very beginning of tau switching into pathological aggregation forms, tau protein is progressively abnormally phosphorylated at several specific amino acid residues (Braak et al., 1994; Augustinack et al., 2002) which contributes to its detaching from MTs and misfolding (Ding et al., 2006). At least, two conformational changes

detected by monoclonal antibodies recognizing discontinuous epitopes have been identified. In pre-tangle neurons, the phosphorylation may act as an inducer of the first conformational change where the extreme N-terminus folds and contacts the MTBD (Carmel et al., 1996; Jicha et al., 1997; Jicha et al., 1999). The sequential tau truncation both at C-terminal site D421 (Guillozet-Bongaarts et al., 2005) and also at N-terminus may facilitate formation of the later conformational change of tau where proline-rich region is in close proximity to MTBD (Ghoshal et al., 2001; Garcia-Sierra et al., 2003) followed by additional cleavage events such as E391 truncation (Skrabana et al., 2004). All these changes in tau molecule seem to be specific for the formation of pathological tau aggregates and contribute to tau fibrillization associated with toxic consequences.

Naturally occurring tau-reactive antibodies

Naturally occurring antibodies (nAbs) of IgG, IgM, and IgA subclasses produced independently of the introduction of foreign antigens constitute approximately two-thirds of serum immunoglobulins (Igs) repertoire. These natural Abs are germline gene-coded Abs occurring in physiological serum characterized by polyspecificity (polyreactivity) and associated with variable, predominantly low, antigen-binding affinities as they are not subjected to affinity maturation and thus contain none or very few somatic mutations (Willis et al., 2013). A subset of natural antibodies generated against various intracellular constituents, plasma proteins, cell-surface antigens, neoantigens, and their altered forms are natural autoantibodies with a wide range of affinity values often cross-reacting with several antigens, including self-antigens (Avrameas, 1991).

Circulating naturally occurring (auto)antibodies are assumed to have many crucial physiological roles. They provide the preliminary innate immune response against exogenous pathogens by several mechanisms, such as activation of the complement system, direct neutralization of pathogens, the formation of antigen-antibody immunocomplexes, and thus modulate the course of onset of infection by participating in nonspecific defense (Ochsenbein and Zinkernagel, 2000). Moreover, their role in immunoregulation has been well documented by binding and clearance of apoptotic cells as well as potentially harmful self-antigens, altered

molecules, to prevent disproportionate activation of other components of immune system. The anti-idiotypic network consistent from nAbs is beneficial in the suppression of an inadequate response of various serum autoantibodies to avoid development of exaggerated immune response leading to autoimmune pathology (Rossi et al., 1989; Kieber-Emmons et al., 2012). Thus, nAbs significantly contribute to the maintenance of normal immune homeostasis. Beside these predominantly beneficial natural antibodies, autoantibodies characteristic for various autoimmune disorders can be formed under pathological conditions when a disbalance in immune system occurs (Scofield, 2004; Lleo et al., 2010). These harmful autoantibodies are almost exclusively monoreactive with higher affinities to particular self-antigens. Several hypotheses explaining how a part of autoantibody repertoire switches into genuine pathogenic autoantibodies have been suggested and still vividly discussed. The dysregulation of physiological immune processes may lead to defective immune clearance of immunocomplexes and their subsequent deposition in various tissues with pathological consequences. Also, aberrant presentation of previously hidden or chemically altered molecules as newly recognized self-antigens to components of the immune system gives rise to autoantibodies with a toxic gain of function. Molecular mimicry, the sharing of common antigenic structures by exogenous pathogens and host molecular structures may cause an inaccurate cross-reactive response of autoantibodies with self-antigens and trigger the chain of events leading to inadequate immunological response and even development of autoimmune diseases, reviewed in (Atassi et al., 2008). Thus, due to the generalized assumption that low-affinity polyreactive natural antibodies have numerous beneficial functions and high-affinity monoreactive autoantibodies appearing in high titers under pathological conditions are harmful; it is still discussed whether the function of serum natural (auto)antibodies directed to brain antigenic entities is more protective or rather pathological in neurological disorders (Levin et al., 2010; Loeffler, 2014).

Circulating naturally occurring tau-reactive antibodies (nTau-Abs) of both IgG and IgM isotype has been detected in serum and CSF of Alzheimer's disease patients as well as in healthy controls (Rosenmann et al., 2006; Fialova et al., 2011; Bartos et al., 2012; Klaver et al., 2017; Krestova et al., 2017; Kuhn et al., 2018). To date, it has not been fully established whether these immunoglobulins are generated as antibodies after the stimulation of immune system by tau protein as a specific antigen or whether they are naturally occurring antibodies mostly with the poly-/cross-reactive profile.

Interestingly, tau protein was confirmed to be present in plasma samples of healthy donors, MCI and AD patients, although in concentrations hundred times smaller than in CSF, with elevated levels in those of AD patients (Zetterberg et al., 2013). Even, tau oligomers which are considered to be the most toxic tau species have been detected in the human serum of healthy donors, MCI and AD patients, with lower levels in MCI samples (Kolarova et al., 2017). Several systems of protein clearance from brain tissue have been known, some of them are appropriate to explain how neuronal intracellularly localized tau protein can occur in peripheral blood circulation, reviewed in (Tarasoff-Conway et al., 2015). Thus, tau protein in various modifications present in the periphery may serve as an antigen stimulating immunological response to produce tau-reactive antibodies. On the other hand, Kuhn et al. observed no significant differences in levels of tau-reactive antibodies in children sera compared to those of adults showing tau-reactive antibodies as to be the pool of naturally occurring antibodies whose formation is mostly antigen-independent (Kuhn et al., 2018). Also, the intrathecal synthesis seems to be a partial source of tau-reactive Abs (Fialova et al., 2011; Bartos et al., 2012).

To evaluate the significance of nTau-Abs under physiological conditions as well as pathological circumstances is a difficult task. In general, their presence in sera of healthy individuals and intravenous immunoglobulin (IVIG) products suggests that they are unlikely to be harmful (Smith et al., 2013). But to date, it can only be speculated whether they have a protective effect against tau pathology, such as participating in clearance of modified and misfolded tau molecules, blocking tau polymerization, degrading tau aggregates, and/or inhibiting tau oligomer neurotoxicity, suggested their specific mechanisms of action in immunotherapy (Schroeder et al., 2016). Hence, from this perspective, alterations in fulfilling their physiological role may significantly contribute to tau pathology. Also, different, more specific tau-reactive Abs may be generated as a result of presentation of disease-specific modified tau forms as newly presenting self-antigens, and contribute to immune imbalance, inflammation and AD progression. In all studies regarding detection of naturally occurring tau-reactive antibodies in sera, relative antibodies levels were determined by enzyme-linked immunosorbent assays (ELISA) in which human recombinant full-length tau (Rosenmann et al., 2006; Kuhn et al., 2018), purified bovine tau (Fialova et al., 2011; Bartos et al., 2012), or tau fragments, 195–213 aa phosphorylated at 202/205 (Rosenmann et al., 2006) and 196-207,

phosphorylated/non-phosphorylated at 199/202, respectively, (Klaver et al., 2017) were applied as assay antigens. Surprisingly, no statistically significant differences were found in levels of sera tau-reactive Abs of IgG isotype between groups of healthy controls and AD patients.

Considering a huge range of variations in physiological and pathological modifications of tau protein, the pool of tau-reactive Abs occurring in body fluids is likely to be constituted from a heterogeneous mixture of antibodies against various tau epitopes with distinct and specific characteristics. Thus, more detailed evaluation of their quality in sense of their character (such as reactivity profile against various tau species, avidity, IgG subclasses distribution, and potential protective effect against tau pathology) should give us much more valuable information about their physiological role as well as their potential contribution in AD development and progression. This challenging topic has become more and more important since a relatively new encouraging strategy for AD therapy is an administration of IVIG products prepared from plasma of healthy donors representing a huge reservoir of natural antibodies. Numerous beneficial immunomodulatory and anti-inflammatory effects of IVIG and their application in the treatment of several immune-mediated neurological disorders has been described (Durandy et al., 2009; Zivkovic, 2016). Unfortunately, the clinical trials phase III with IVIG in AD treatment has not supported the promising results obtained in the initial phases I and II (Relkin et al., 2014). Despite these disappointing results so far, several other IVIG clinical trials are ongoing (Cummings et al., 2017). Moreover, a considerable effort is devoted to the investigation of the potential mechanisms of action of IVIG and better characterization of AD-specific antibodies occurring in various IVIG products.

To the best knowledge, to date, five studies regarding the presence of tau-reactive Abs in IVIG products have been published. Loeffler´s group investigated mostly variations in levels of naturally occurring anti-tau Abs in different IVIG products. The first evidence of presence of naturally occurring Abs directed to recombinant human full-length tau in three IVIG products was published in 2013 (Smith et al., 2013), followed by determination of levels of Abs against various tau fragments representing tau regions differing in effect on tau aggregation (Smith et al., 2014) and AD-specific phosphorylation (Loeffler et al., 2015). In all three works, all tested IVIG products contain nAbs reactive to tau antigens and their levels significantly varied among some IVIG products, which could reflect variations in specific antibody concentrations between the

donor pools used for plasma collection and/or the procedures used for antibody isolation and purification (Lejtenyi and Mazer, 2008; Smith et al., 2013), Also, the artefactual phenomenon for low-affinity antibodies determined by *in vitro* binding assays as was suggested by (Cattepoel et al., 2016) may be a source of these variations. Polyvalent binding of immunoglobulins, which has to be an account in order to determine specific natural antibody concentrations in complex samples such as serum, plasma, and IVIG, was also discussed (Loeffler and Klaver, 2017).

As an alternative approach of AD treatment is suggested the use of AD-specific IVIG preparations with all their beneficial immunomodulatory and anti-inflammatory effects enriched with polyclonal AD antigen-specific antibodies (Loeffler, 2014). To support this approach, isolation of these AD protein-specific nAbs from IVIG products is needed to characterize whether they may have a potential to reverse the protein pathology. More work in this field has been done with nAbs directed to Aβ peptides (reviewed in a book chapter: Bach and Dodel, 2012). But an interest in tau-based immunotherapy, mostly supported by the fact that tau pathology better correlates with AD progression and may be independent on Aβ accumulation, has been increasing (Arriagada et al., 1992; Nelson et al., 2012). The first data regarding *in vitro* characterization of tau-reactive nAbs isolated from IVIG product was published by our group in 2016 (Hromadkova et al., 2015). The comparison of their reactivity profile with nTau-Abs isolated from pooled plasma samples from AD patients and healthy controls was followed (Krestova et al., 2017). Both studies revealed that isolated tau-reactive Abs can recognize abnormally modified tau forms and vary in many characteristics. Effects of these tau-specific nAbs on prevention of formation and/or clearance of pathological tau forms have to be evaluated in the further investigation with cell-based and animal model systems.

AIMS OF THE THESIS

The experimental part of this thesis could be divided into two main aims.

The first goal was to evaluate tau-reactive antibodies isolated from plasma samples, including IVIG product. Within this aim, the specific properties of tau protein as a natively unfolded protein were also investigated for further applications in mass spectrometry analysis.

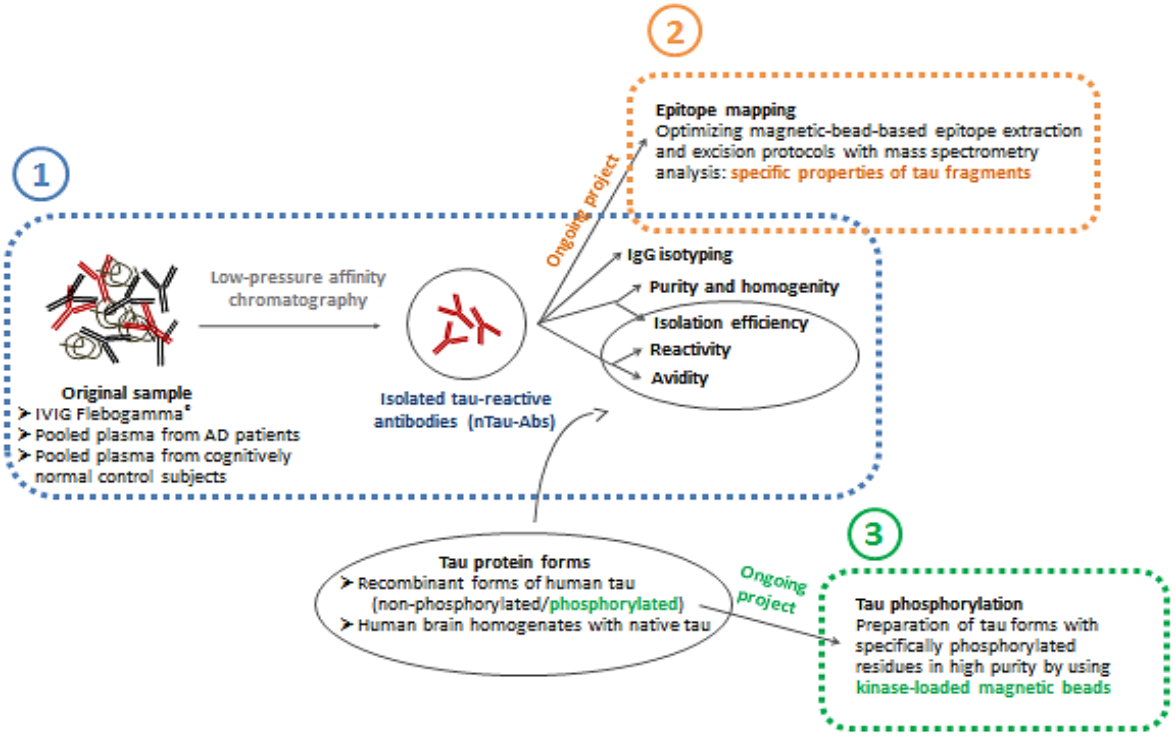
The second task was the preparation of tau protein with defined phosphorylated sites in high purity for *in vitro* applications.

To fulfill these tasks, specific sub-goals were set

1) To evaluate the basic characteristics and reactivity of tau-reactive antibodies isolated from different pooled plasma samples utilizing various tau protein forms.

2) To optimize methods of epitope mapping considering specific aggregation-prone properties of tau protein enhanced by *in vitro* fragmentation.

3) To prepare kinase-loaded magnetic beads as a reusable system for sequential *in vitro* tau phosphorylation to produce highly pure defined product for further applications.



Scheme of the main aims of the thesis

LIST OF PUBLICATIONS

PUBLICATIONS IN PEER-REVIEWED JOURNALS WITH IF

PUBLICATIONS RELATED TO THIS THESIS (INCLUDED *IN EXTENSO*)

- **Hromadkova, L.**, Kupcik, R., Jankovicova, B., Rousar, T., Ripova, D., Bilkova, Z. (2016). Difficulties associated with the structural analysis of proteins susceptible to form aggregates: The case of Tau protein as a biomarker of Alzheimer's disease. *Journal of Separation Science*, *39*(4), 799-807.
  (Impact factor in 2016: 2.557)

- **Hromadkova, L.**, Kolarova, M., Jankovicova, B., Bartos, A., Ricny, J., Bilkova, Z., Ripova, D. (2015). Identification and characterization of natural antibodies against tau protein in an intravenous immunoglobulin product. *Journal of Neuroimmunology*, *289*, 121-129.
  (Impact factor in 2015: 2.536)

- Krestova, M., **Hromadkova, L.**, Bilkova, Z., Bartos, A., Ricny, J. (2017). Characterization of isolated tau-reactive antibodies from the IVIG product, plasma of patients with Alzheimer's disease and cognitively normal individuals. *Journal of Neuroimmunology*, *313*, 16-24.
  (Impact factor in 2016: 2.720)

- **Hromadkova, L.**, Kupcik, R., Vajrychova, M., Prikryl, P., Charvatova, A., Jankovicova, B., Ripova, D., Bilkova, Z., Slovakova, M. (2018). Kinase-loaded magnetic beads for sequential in vitro phosphorylation of peptides and proteins. *Analyst*, *143*(2), 466-474.
  (Impact factor in 2016: 3.885)

PUBLICATIONS UNRELATED TO THIS THESIS

- Schedin-Weiss, S., Inoue, M., **Hromadkova, L.**, Teranishi, Y., Yamamoto, N. G., Wiehager, B., Bogdanovic, N., Winblad, B., Sandebring-Matton, A., Frykman, S., Tjernberg, L. O. (2017). Monoamine oxidase B is elevated in Alzheimer disease neurons, is associated with γ-secretase and regulates neuronal amyloid β-peptide levels. *Alzheimer's Research & Therapy*, *9*(1), 57. (Impact factor in 2016: 6.154)

- **Hromadkova, L.**, Zarybnicka, L., Vavrova, J., Bilkova, Z., & Sinkorova, Z. (2014). The impact of pre-analytical treatment and sorting on human neutrophil function. *Veterinární Medicína*, *59*(11). (Impact factor in 2014: 0.639)

PUBLICATIONS RELATED TO THIS THESIS IN PEER-REVIEWED JOURNALS WITHOUT IF (NOT INCLUDED *IN EXTENSO*)

- Jankovičová, B., **Hromádková, L**., Kupčík, R., Kašparová, J., Řípová, D., Bílková, Z. (2014). Quality evaluation of monoclonal antibodies suitable for immunomagnetic purification of native tau protein. *Scientific Papers of the University of Pardubice Series A, Faculty of Chemical Technology*, *20*, 147-163.

- Jankovicova, B., Svobodova, Z., **Hromadkova, L.**, Kupcik, R., Ripova, D., Bilkova, Z. (2015). Benefits of Immunomagnetic Separation for Epitope Identification in Clinically Important Protein Antigens: A Case Study Using Ovalbumin, Carbonic Anhydrase I and Tau Protein. *Universal Journal of Biomedical Engineering*, *3*, 1-8.

CHAPTER IN BOOK

- Krestova, M., **Hromadkova, L.**, Ricny, J. (2017). Purification of Natural Antibodies against Tau Protein by Affinity Chromatography. *Natural Antibodies: Methods and Protocols*, 33-44.

PUBLICATIONS *IN EXTENSO*

Publication I:

Difficulties associated with the structural analysis of proteins susceptible to form aggregates: The case of Tau protein as a biomarker of Alzheimer's disease.

Hromadkova, L., Kupcik, R., Jankovicova, B., Rousar, T., Ripova, D., Bilkova, Z., Journal of Separation Science, 2016, 39(4), 799-807.

Stated contributions of the author: Study design. Experimental part (covalent binding of proteases to magnetic beads, tau fragmentation, preparation of immunosorbents, epitope extraction, Thioflavin S assay). Manuscript design and writing.

Lenka Hromadkova[1,2,3]
Rudolf Kupcik[3]
Barbora Jankovicova[3]
Tomas Rousar[3]
Daniela Ripova[1]
Zuzana Bilkova[3]

[1]Department of Neurobiology and AD Center, National Institute of Mental Health, Klecany, Czech Republic
[2]Faculty of Science, Charles University in Prague, Prague, Czech Republic
[3]Department of Biological and Biochemical Sciences, University of Pardubice, Pardubice, Czech Republic

Received September 17, 2015
Revised November 24, 2015
Accepted November 26, 2015

Research Article

Difficulties associated with the structural analysis of proteins susceptible to form aggregates: The case of Tau protein as a biomarker of Alzheimer's disease

Mass spectrometry coupled with bioaffinity separation techniques is considered a powerful tool for studying protein interactions. This work is focused on epitope analysis of tau protein, which contains two VQIXXK aggregation motifs regarded as crucial elements in the formation of paired helical filaments, the main pathological characteristics of Alzheimer's disease. To identify major immunogenic structures, the epitope extraction technique utilizing protein fragmentation and magnetic microparticles functionalized with specific antibodies was applied. However, the natural adhesiveness of some newly generated peptide fragments devalued the experimental results. Beside presumed peptide fragment specific to applied monoclonal anti-tau antibodies, the epitope extraction repeatedly revealed inter alia tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317 containing the fibril-forming motif 306-VQIVYK-311. The tryptic fragment pro-aggregation and hydrophobic properties that might contribute to adsorption phenomenon were examined by Thioflavin S and reversed-phase chromatography. Several conventional approaches to reduce the non-specific fragment sorption onto the magnetic particle surface were performed, however with no effect. To avoid methodological complications, we introduced an innovative approach based on altered proteolytic digestion. Simultaneous fragmentation of tau protein by two immobilized proteases differing in the cleavage specificity (TPCK-trypsin and α-chymotrypsin) led to the disruption of motif responsible for undesirable adhesiveness and enabled us to obtain undistorted structural data.

Keywords: Epitope extraction / Mass spectrometry / Nonspecific sorption / Tau protein / Thioflavin S assay
DOI 10.1002/jssc.201501045

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Recently, many clinicians and biochemists are interested in the structure and immunogenic properties of proteins, which seem to be a key element in the formation of aggregates that threaten healthy tissue. This interest is also due to the constantly growing number of patients suffering from various neurodegenerative diseases, which have been in common classified as conformational disorders containing aggregates with a predominance of a single protein/peptide type in brain tissues [1–3]. Alzheimer's disease (AD), the most common neurodegenerative disease, is ranked among tauopathies due to the formation of intracellular aggregates based on paired helical filaments (PHF) consisting of modified and misfolded tau protein [4]. In the microtubule-binding (MTB) domain of tau protein, there are hot-spot amino acid sequences susceptible to form β-sheet and α-helix structures and to initiate aggregation [5]. In association with tau pro-aggregation properties, much attention has been dedicated especially to the two

Correspondence: Professor RNDr. Zuzana Bilkova, Ph.D, Department of Biological and Biochemical Sciences, University of Pardubice Studentska 573, HB/C 532 10 Pardubice, Czech Republic
E-mail: Zuzana.Bilkova@upce.cz

Abbreviations: ACN, acetonitrile; **AD**, Alzheimer's disease; **BApNA**, *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; **CHCA**, α-cyano-4-hydroxycinnamic acid; **DAHC**, diammonium hydrogen citrate; **DHB**, 2,5-dihydroxybenzoic acid; **DTT**, dithiothreitol; **EDAC**, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; **IAA**, iodoacetamide; **mAb**, monoclonal antibody; **MES**, 2-(*N*-morpholino)ethanesulfonic acid; **MPs**, magnetic particles; **MT**, microtubule; **PHF**, paired helical filaments; **RT**, room temperature; **sulfo-NHS**, *N*-hydroxysulfosuccinimide sodium salt; **SUPHEPA**, *N*-succinyl-L-phenylalanine-*p*-nitroanilide; **ThS**, Thioflavin S; **TPCK**, L-1-tosylamido-2-phenylethyl chloromethyl ketone

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

regions at the N-termini of R2 and R3 repeats within MTB domain, PHF6* (275-VQIINK-280) and PHF6 (306-VQIVYK-311) [6]. The significant portion of AD therapeutic research is focused on antibody response with the potential to abolish the progression of tau pathology, mostly by preventing the oligomerization and the PHF formation.

The substantial effort is incurred for the development of antibodies distinguishing among various tau protein forms (phosphorylated, truncated, misfolded, pre-aggregated, aggregated, etc.). Thus, the verification of such anti-tau antibody specificity is crucial and different epitope mapping approaches are needed [7, 8]. In recent years, epitope mapping techniques for the identification of continuous epitopes have achieved considerable development due to peptide library technologies [9]. Even so, the approaches based on proteolytic fragmentation of antigens, such as epitope extraction and epitope excision combined with MS detection are still favored and affordable alternatives [10–12]. In the basic protocol of epitope extraction technique, the target protein is digested by a proteolytic enzyme(s) and generated fragments are subsequently incubated with specific antibodies (monoclonal, polyclonal, patient) bound to the solid phase. After washing steps, the fragments interacting with binding sites of antibodies are eluted and identified by MS [13, 14]. MS combined with magnetic-bead-based epitope mapping is the most efficacious methodological approach for such analysis [15–18].

To use this technique for structural analysis of intrinsically disordered proteins containing pro-aggregated motifs, it is necessary to take into account their increased adhesivity to the solid surface. The handling tau protein, which is a typical representative of such proteins, is tricky during analytical and separation steps [19,20]. In this paper, a monoclonal antibody with defined immunogenic epitope was applied as a model system to verify the validity of epitope extraction method with tau protein and to show difficulties connected with tryptic tau peptides having attributes associated with aggregation and hydrophobicity even further exacerbated. The advanced epitope extraction procedure using two proteolytic enzymes differing in cleavage specificity offers the smart solution. This paper emphasizes the difficulties linked with a structural analysis of proteins susceptible to form aggregates and highlights the possible way how to obtain relevant data.

2 Materials and methods

2.1 Reagents and equipment

Recombinant tau protein (human 2N4R variant) was supplied by Enzo Life Sciences (Farmingdale, NY, USA). Synthetic peptide with amino acid sequence 299-HVPGGGSVQIVYKPVDLSK-317 was obtained from Apronex (Prague, Czech Republic). Sera-Mag carboxylate-modified Magnetic SpeedBeads (0.816 μm, hereinafter referred to as MPs) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Monoclonal anti-tau antibody clone 7E5 was purchased from AJ Roboscreen (Leipzig, Germany). BSA, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.22.2, 12,700 IU/mg solid), α-chymotrypsin from bovine pancreas (EC 3.4.21.1, 57.24 IU/mg solid), dithiothreitol (DTT), iodoacetamide (IAA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), Tris, *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BApNA), Thioflavin S (ThS), heparin sodium salt from porcine intestinal mucosa, *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SUPHEPA), 2-(*N*-morpholino)ethane sulfonic acid (MES), acetonitrile (ACN), ethylene glycol, benzamidine, and diammonium hydrogen citrate (DAHC) were produced by Sigma–Aldrich (St. Louis, MO, USA). *N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) and TFA were obtained from Fluka (Buchs, Switzerland). A Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL, USA). M-PEG-NH2 (30 kDa) was obtained from Laysan Bio (Huntsville, AL, USA) and m-PEG-NH2 (2 kDa) from Fluka (Buchs, Switzerland). The MS matrices α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were from LaserBio Labs (Sophia-Antipolis, France). All other chemicals were of reagent grade.

A magnetic separator (Dynal, Carlsbad, CA, USA), high-resolution MALDI tandem mass spectrometer linear trap quadrupole (LTQ) Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA), and SpeedVac RVC 2–18 from Christ (Osterode am Harz, Germany) connected to a vacuum pump from KNF Neuberger (Freiburg, Germany) were used. POROS Oligo R3 reversed-phase material was purchased from Life Technologies (Carlsbad, CA, USA). GELoader tips were from Eppendorf (Hamburg, Germany). RP Ascentis® Express Peptide ES-C18, 2.7 μm particles were from Sigma–Aldrich (St. Louis, MO, USA). A 96-well plate Nunclon Delta Surface was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Microplate reader Infinite M200 was obtained from Tecan Trading (Mannedorf, Switzerland).

2.2 Proteolytic digestion of tau protein

TPCK-treated trypsin or α-chymotrypsin was covalently bound to the MPs using a carbodiimide one-step method while adding EDAC (120 mM) and sulfo-NHS (20 mM) that was adapted from [21]. The activity of immobilized enzymes was determined in a 96-well plate by hydrolysis of chromogenic substrates: BApNA for TPCK-treated trypsin [22] and SUPHEPA for α-chymotrypsin [23].

Tau protein was unfolded by reductive alkylation using DTT and IAA [24] in 50 mM ammonium bicarbonate solution. Unfolded protein was digested for 2 h at 37°C under mild stirring by immobilized TPCK-trypsin or by immobilized TPCK-trypsin and α-chymotrypsin simultaneously in molar ratios E/S of 1:20 for both enzymes. The mixture of generated tau fragments was used for epitope extraction. The efficiency of enzymatic digestion was verified by Tricin/SDS-PAGE electrophoresis [25] and by MALDI-Orbitrap MS.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

2.3 Epitope extraction procedure

For capturing specific tau fragments, immunosorbent was prepared by immobilization of monoclonal anti-tau antibody (20 μg, clone 7E5) to MPs (500 μg) preactivated by EDAC/sulfo-NHS chemistry [21]. The control MPs coated by BSA (500 μg) were also prepared by the same chemistry. The amount of antibody bound to magnetic particles was estimated from the protein mass balance among the initial solution, the binding fraction, and the first washing fraction by BCA test according to the manufacturer´s instructions. In some cases, the surface of MPs was modified by ethanolamine/Tris according to [26] or by PEG (2 or 30 kDa) according to [27].

Tryptically digested recombinant tau protein (5 μg per reaction) was added to washed biofunctionalized MPs (500 μg). Immunoprecipitation was carried out for 3 h at room temperature (RT) under mild stirring. Washing steps followed: 15 times with 1 mL of 0.1 M phosphate buffer pH 7.0 (containing 0.2 M and then 1 M NaCl), three times with 1 mL of 0.01 M phosphate buffer pH 7.0, and finally twice with 1 mL of ultrapure water. Elution of immunoprecipitated peptides was performed three times using 0.2 mL 0.05% TFA for 20 min at RT under mild stirring. Pooled eluted fractions were dried in the SpeedVac and analyzed by MALDI-Orbitrap MS.

The incubation with 8 M urea as an additional step to suppress protein–protein interactions was inserted after the last washing cycle with 0.01 M phosphate buffer pH 7.0 for 10 min and the washing with 1 mL of ultrapure water followed five times.

2.4 MALDI-MS analysis

2.4.1 Desalting and concentration of peptide samples

Custom-made chromatographic reversed-phase (POROS Oligo R3) microcolumns used for desalting and concentration of peptides were prepared using GELoader tips as previously described [28]. Dried peptides (5 μL of elution fractions) were diluted in 0.1% TFA and applied onto POROS Oligo R3 microcolumns using gentle air pressure. The columns were washed with 15 μL of 0.1% TFA. The retained peptides were directly eluted onto MALDI-target with 1 μL CHCA (5 mg/ml in 60% ACN/0.1% TFA + 2 mM DAHC) or DHB solution (10 mg/mL in 50% ACN/0.1% TFA). A MALDI-Orbitrap MS measured in positive mode with a resolution of 100 000 full width at half maximum (FWHM) at m/z 400. Peptides were identified using high mass accuracy and using MS/MS analysis of selected peaks with manual interpretation.

2.4.2 RP-LC separation using a simple microgradient device

A home-made RP capillary column (21 mm long, 250 μm ID) packed in FEP tubing was prepared as previously described [29] with slight modifications. A piece of capillary (360 μm od and 50 μm id) 15 mm in lengths was inserted into FEP tubing (1/16´´ OD × 0.25 mm ID) 35 mm long and column was packed with core–shell C18 particles (Ascentis® Express peptide ES-C18, 2.7 μm). This reversed-phase microcolumn was used for chromatographic separations of the tryptic digest of tau protein before MALDI-Orbitrap MS detection. The column was first wetted with 80% ACN/0.1% TFA and then equilibrated with 20 μL of 2% ACN/0.1% TFA v/v. Tau protein (20 pmol) digested by immobilized TPCK-trypsin or simultaneously by immobilized TPCK-trypsin and α-chymotrypsin was aspirated into a 25 μL microsyringe and loaded onto the column. The microsyringe was filled consecutively by six ACN/0.1% TFA mobile phases with decreasing ACN content: 4 μL of 35, 28, 21, 14, 7, 2% ACN (all v/v). Peptides were eluted using a nonlinear gradient of acetonitrile/water mixture (2–35%) containing 0.1% TFA directly onto 24 MALDI spots. Eluted samples were covered with 0.7 μL of matrix solution–CHCA (5 mg/mL in 60% ACN/0.1% TFA + 2 mM DAHC) or DHB (10 mg/mL in 50% ACN/0.1% TFA) and then analyzed by MALDI-Orbitrap MS. After separation, the entire system was washed consecutively with 20 μL of 80% ACN/0.1% TFA v/v and 20 μL of pure ACN and then separation was repeated with a blank. The results were confirmed with synthetic tau peptide analogue 299-HVPGGGSVQIVYKPVDLSK-317. A MALDI-Orbitrap MS was used in a positive mode with resolution 60 000 FWHM at m/z 400.

2.5 ThS fluorescence measurement

The synthetic peptide 299-HVPGGGSVQIVYKPVDLSK-317 was dissolved in 1 mM DTT in PB pH 7.0. To minimize the formation of disulfide bonds, the mixture was incubated 30 min at 37°C. The 15 min sonication followed to reduce aggregated intermediates before the assay. Fibrillization of 20 μM synthetic tau peptide was carried out in epitope extraction solutions and 25% ACN/0.1% TFA containing 10 μM ThS at RT in the presence or absence of 10 μM heparin sodium salt. The fluorescence signal was followed at 490 nm (emission bandwidth of 20 nm) with excitation at 440 nm (excitation bandwidth of 9 nm). Kinetics was determined at 20 min intervals for 24 h. Each sample and the corresponding blank were measured in triplicate. The corresponding controls (the absence of synthetic peptide) were subtracted.

3 Results

One of the procedures enabling identification of the main continuous immunogenic epitopes of the analyzed proteins is known as epitope extraction and consists of two steps: enzymatic fragmentation of protein and immunoextraction of desired peptides with epitopes by specific antibodies [15]. For final epitope identification, MS is applied. This strategy was applied for identification of the main immunogenic

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

structure of tau protein, a typical representative of proteins with aggregation motifs.

3.1 Enzymatic cleavage of tau protein

Tau protein (human tau-F isoform, 441 amino acids) was cleaved either by immobilized TPCK-trypsin (995.68 IU/mg of MPs) or simultaneously by immobilized TPCK-trypsin and α-chymotrypsin (59.3 IU/mg of MPs) according to a standard protocol (see Section 2.2). The generated mixture of tryptic fragments represents the starting material for epitope extraction procedure. Tricin/SDS-PAGE electrophoresis provided us information about the purity and homogeneity of recombinant tau protein to be digested (Supporting information, Fig. SI1).

The digestion was efficient, 63.3% sequence coverage was achieved, based on peptide mass fingerprinting search using MS-Fit tool against NCBI database (HUMAN taxonomy). Mass tolerance was set at 10 ppm and specific cleavage with trypsin and chymotrypsin (C-term to K/R/F/Y/W, not before P) with up to two missed cleavages was used for database search. The occurrence of the fragment 299-HVPGGGSVQIVYKPVDLSK-317 (theoretical *m/z* 1980.0912) generated by immobilized TPCK-trypsin digestion of tau protein as well as its cleavage products by α-chymotrypsin were identified by MS analysis (Supporting information, Fig. SI2). Table 1 provides information about these fragments generated in silico enzymatic digestion (C-term to K/R/F/Y/W/M/L, not before P) with calculated theoretical *m/z* values which were used for the proper identification of specific peptides in trypsin/chymotrypsin digests. In addition to the specific fragments stated in Table 1, the semi-specific peptide 301-PGGGSVQIVY-310 originating from 299-HVPGGGSVQIVYKPVDLSK-317 was also observed in mass spectra (Supporting Information Fig. SI2). The identity of fragments was confirmed by MS/MS analysis (data not shown).

3.2 Immunoextraction of tau peptides

To implement the epitope extraction procedure for tau protein, the immunosorbent with anti-tau mAb clone 7E5 was prepared. The amount of immobilized IgG was 16.41 μg/0.5 mg of the carrier (estimated by BCA assay). The sequence of epitope reacting with monoclonal anti-tau mAb (7E5) declared by the manufacturer is 155-RGAAPPGQKGQA-166; theoretical epitope-containing tryptic and chymotryptic fragments, which should be observed in elution fractions, are summarized in Supporting Information Table SI1.

Tau fragments generated by TPCK-trypsin or simultaneously by TPCK-trypsin and α-chymotrypsin were applied to the specific immunosorbent or unmodified/BSA-modified MPs (negative controls). After the immunocapturing, washing steps with increasing ionic strength of NaCl (0.2–1.0 M) were performed to remove all tau tryptic fragments that did not react with the specific mAb. Elution of immunocaptured tryptic peptides by 0.05% TFA followed. All acquired fractions were subsequently analyzed by MALDI-Orbitrap MS.

Regardless of the applied immunosorbent specificity, we repeatedly found in MS spectra of elution fractions a tryptic fragment corresponding to tau amino acid sequence 299-HVPGGGSVQIVYKPVDLSK-317 (theoretical *m/z* 1980.0912; Fig. 1A). We observed this fragment also in fractions eluted from the unmodified carrier (negative control, data not shown). Due to its high relative intensity, this adhesive fragment negatively affected the interpretation of MS spectra.

Therefore, we introduced several conventional alterations to avoid this nonspecific adsorption. An additional coating of MPs surface by inert protein BSA was applied, but the adhesive fragment still occurred in all elution fractions (Supporting information, Fig. SI3). Even the brief incubation with 8 M urea during the washing phase, a step commonly used to prevent nonspecific protein–protein interactions, was not effective (Fig. 1B, Supporting Information Fig. SI3B). To minimize the adhesivity of magnetic-bead-based carriers, we blocked the remaining reactive groups on the MPs surface by ethanolamine/Tris or we reduced the hydrophobicity of beads by polymers m-PEG-NH_2 of 2 or 30 kDa molecular weight. Epitope extraction experiments performed with these enhanced carriers and synthetic peptide analogue 299-HVPGGGSVQIVYKPVDLSK-317 did not prevent the occurrence of the adhesive fragment in elution fractions (Supporting information Fig. SI4). Moreover, elution of synthetic peptide analogue using 0.05% TFA from MPs modified by m-PEG-NH_2 (2 kDa) was accompanied by the excessive release of contaminating PEG residues (Fig. SI4A).

As an alternative step, we tested the effect of tau protein fragmentation simultaneously by two immobilized proteases, TPCK-trypsin and α-chymotrypsin. Tryptic digestion was maintained to obtain fragments of a length appropriate for MS analysis. Chymotrypsin was selected based on its cleavage preferences that enable to cleave peptide bond 310-YK-311 solely in the PHF6 minimal interaction motif. This multi-enzymatic approach appeared to be efficient for the elimination of the non-specifically interacting tau fragment in eluted fractions, whereby specific fragments encompassing the epitope (*m/z* 1423.7461 and 1249.6355 for mAb clone 7E5) remained detectable in the spectrum (Fig. 1C), and distinctly improved the spectra interpretation. All modifications of epitope extraction protocol for tau protein are summarized in Scheme 1.

3.3 RP-LC separation of tau fragments

RP-LC separation using a simple microgradient device was the next procedure wherein we observed the inadequate adhesivity of 299-HVPGGGSVQIVYKPVDLSK-317 fragment. The unusual behavior of adhesive peptide can be demonstrated by its permanent leakage from RP-LC column during separation of tryptic fragments observed in the 9th elution

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Basic characteristics of adhesive tau fragment generated after tryptic digestion and newly-formed fragments after tryptic/chymotryptic digestion

Applied immobilized enzymes	Generated peptide sequence	$[M+H]^+$	Missed cleavage	pI
TPCK-trypsin	299-HVPGGGS**VQIVYK**PVDLSK-317	1980.0912	0	8.5
TPCK-trypsin + α-chymotrypsin	299-HVPGGGS**VQIVYK**PVDL-315	1764.9643	1	6.7
	299-HVPGGGS**VQIVY**-310	1212.6371	0	6.7
	301-PGGGS**VQIVY**-310 *	976.5098	0	6.0
	311-**K**PVDLSK-315	786.4720	1	8.6
	311-**K**PVDL-315	571.3450	0	5.8

*Semi-specific sequence experimentally verified by MS/MS analysis (data not shown).

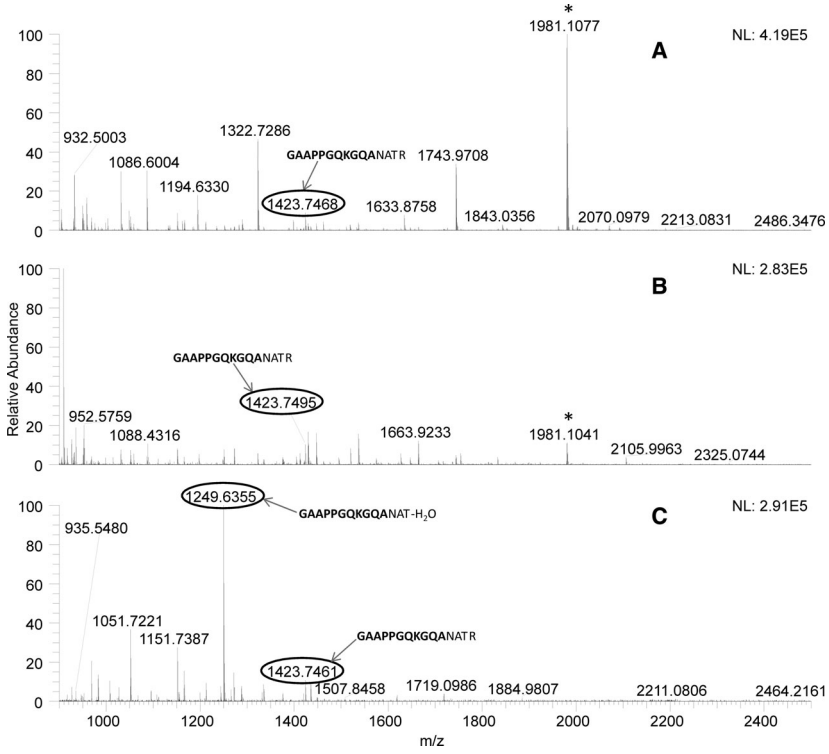


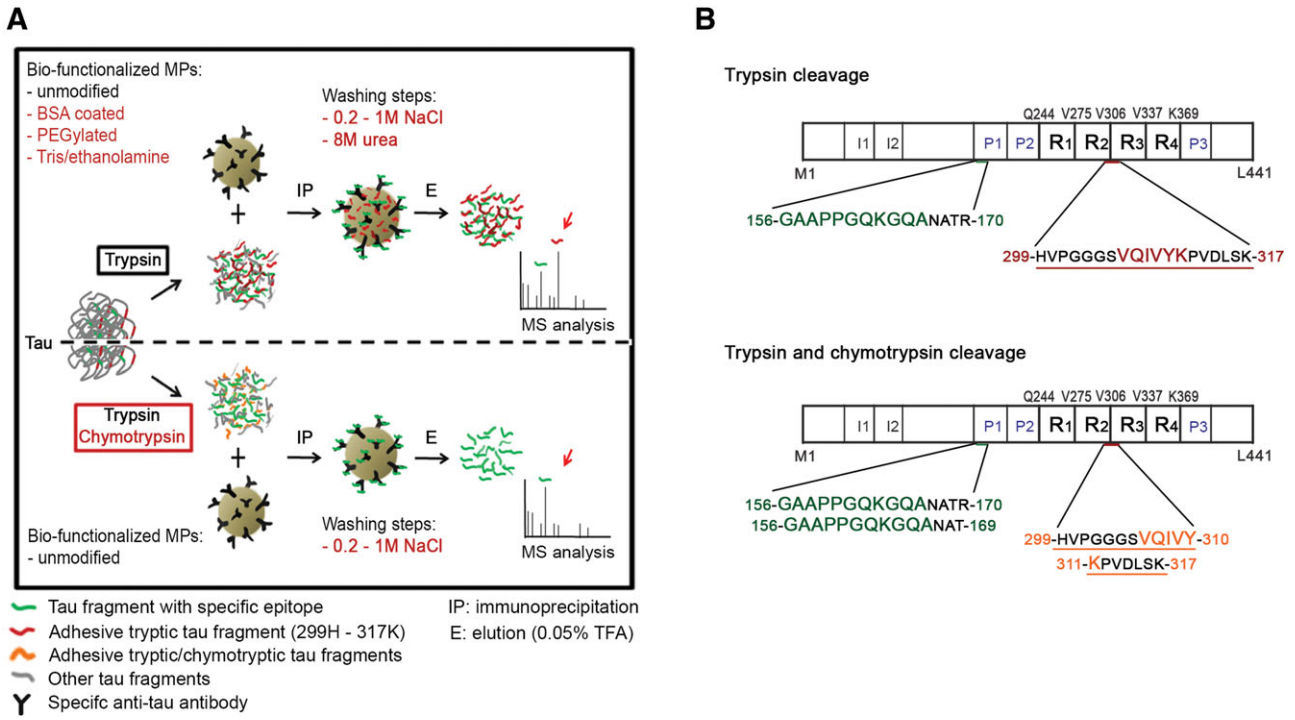
Figure 1. (MALDI)-Orbitrap spectra of elution fractions obtained by epitope extraction using 7E5 monoclonal antibodies in combination with: (A) tau protein (human tau-F isoform) digested with immobilized TPCK-trypsin, (B) tau protein (human tau-F isoform) digested with immobilized TPCK-trypsin and an additional 8 M urea washing, and (C) tau protein (human tau-F isoform) digested with immobilized TPCK-trypsin and α-chymotrypsin. Peptides belonging to adhesive tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317 are marked with an asterisk. Amino acid sequence 156-GAAPPGQKGQANATR-170 with theoretical *m/z* 1423.75 (circled) contains the epitope 155-RGAAPPGQKGQA-166 specific to mAb, clone 7E5.

fraction (Fig. 2A) and persistent to the 24th elution fraction (data not shown). Even after the intensive washing and final regeneration of the chromatographic column, the adhesive peptide was still identified in all eluates. In contrast, after the digestion of tau by immobilized TPCK-trypsin and α-chymotrypsin simultaneously, the newly formed peptides corresponding to 299-HVPGGGSVQIVYKPVDLSK-317 (for *m/z* see Table 1) have lost the adhesive properties of the original fragment and disappeared (Fig. 2B).

3.4 Thioflavin S assay

Protein aggregation and clustering are also mentioned in relation to nonspecific protein–surface adsorption phenomenon [30]. Thus, a ThS assay was performed for monitoring the aggregation behavior that could be caused by the minimal interaction motif PHF6 (306-VQIVYK-311) [31]. The principle of this method is based on non-covalent probing of β-sheet structures by ThS detected as an increased fluorescence signal. To evaluate aggregation predispositions of the tryptic tau fragment 299-HVPGGGSVQIVYKPVDLSK-317, a synthetic peptide analogue was used. These fibrillization experiments were performed in working solutions same as for the epitope extraction procedure (0.1 M PB buffer pH 7.0, 0.1 M PB buffer pH 7.0 with 0.2 M/1 M NaCl, 0.01 M PB buffer, ultra-pure water and 0.05% TFA) and in one of the eluent solutions used in microscale RP-LC (25% ACN/0.1% TFA), with or without heparin as an inducer. The aggregation behavior of tau fragment was observed both in the presence and absence of heparin with differences in the β-sheet assembly rate (ThS fluorescence at 490 nm) (Fig. 3). The presence of NaCl in 0.1 M phosphate buffer pH 7.0 reduces the fragment assembly (Fig. 3B and C). The acidic solutions show much higher ThS fluorescence in comparison to washing solutions in the presence of heparin, but the self-assembly

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. (A) Epitope extraction technique with additional steps (text in red color) to suppress the non-specific adsorption of the tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317. In the upper half of the scheme A, the conventional approaches for sorption suppression are illustrated. In the lower half of the scheme A, our innovative approach utilizing two immobilized enzymes is represented. The red arrows show the location of the adhesive fragment in MS spectra. (B) The simplified figure of human full-length tau protein (htau40) with the most important regions (I – insert domain, P – proline-rich domain, R – repeat domain). The tryptic cleavage and tryptic/chymotryptic cleavage sites are illustrated for the fragment with epitope 155-RGAAPPGQKGQA-166 corresponding to mAb clone 7E5 and the adhesive tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317 occurring in MS spectra.

(no inducer added) was not proven in this case (Fig. 3F, dashed lines).

4 Discussion

Specific properties of a protein observed *in vitro* are naturally associated with their behavior *in vivo* and may contribute to the pathology–morphological processes causing various diseases (e.g., tauopathies, synucleinopathies). However, these characteristics may be responsible for difficulties in numerous analytical method; for example in epitope extraction, a technique using immobilized proteases and carrier with covalently bound specific antibodies enabling identification of the main immunogenic structures of studied proteins. As has been mentioned previously in the case of tau protein we can face unexpected difficulties. Within our epitope extraction experiments performed with anti-tau mAb clone 7E5 (declared specificity 155-RGAAPPGQKGQA-166), we expected one major peak with the highest relative intensity in MS spectra corresponding to peptide fragment containing specific epitope. However, on the contrary, we identified the adhesive fragment 299-HVPGGGSVQIVYKPVDLSK-317 containing PHF6 (306-VQIVYK-311) motif as the most intense peak complicating the interpretation of our results. Even though several different types of monoclonal and polyclonal anti-tau antibodies were used for epitope extraction approach, the adhesive fragment was identified in final MS spectra despite epitope specificity of the antibody (data not shown). As we have discovered this fragment had already been observed as a high-intensity peak in MS spectra in other studies (e.g., Refs. 32,33). To eliminate the distorted results due to the increased non-specific adhesivity of the contaminating peptide, three additional approaches were tested: (i) increased salt molarity of washing solution and additional incubation with chaotropic agent, (ii) surface solid phase treatment to increase the biocompatibility of carrier and (iii) simultaneous fragmentation of tau protein by two proteases differing in specificity. In the case of tau protein, we demonstrated that the conventional approaches (i, ii) how to suppress non-specific sorption on MPs surface were not sufficient [26,27,34]. Moreover, the PEGylation of MPs in epitope extraction protocol using the acidic elution does not bring into any effect and is not convenient due to the polymer washout (Supporting information Fig. SI4).

Repeatedly observed non-specific adsorption of the adhesive peptide on MPs surface led us to the detailed investigation of its characters. We focused mainly on its hydrophobic and pro-aggregation properties. One of the most important features of tau as a naturally unfolded protein is low

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

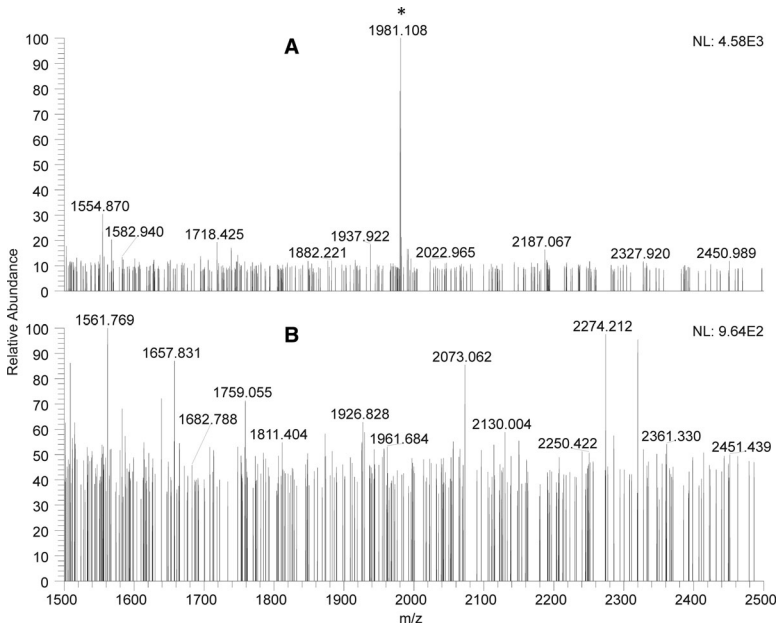


Figure 2. (MALDI)-Orbitrap spectra of the ninth RP-LC separation fraction of: (A) the second washing cycle after RP-LC separation of tau protein (human tau-F isoform) digested by immobilized TPCK-trypsin using a simple microgradient device, and (B) the second washing cycle after RP-LC separation of tau protein (human tau-F isoform) digested by immobilized TPCK-trypsin combined with immobilized α-chymotrypsin using a simple microgradient device. The adhesive tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317 is marked with an asterisk.

content of the hydrophobic amino acids I, W, L, F, and V altogether amounting to 15% [35], but the 13-residue stretch 306-VQIVYKPVDLSKV-318 is considered to be the most hydrophobic region of the 441-residue tau protein molecule [36]. Moreover, tau protein's high surface activity has been proposed as the reason for its adsorption propensity at the hydrophobic air/water interface [37]. Perret-Liaudet and coworkers published a study about the effect of the used collection tube types on the concentration of CSF biomarkers. The ratio of hydrophobic amino acid residues to protein/peptide length may significantly affect the adsorption predisposition as illustrated the conclusions of their paper [38]. We set up a microscale RP-LC experiment using the core–shell C18 solid phase for an indirect testing of the hydrophobic character of the adhesive fragment [39]. We repeatedly confirmed its hydrophobic behavior by exhibiting the greatest affinity to the hydrophobic C18 solid phase. MS spectra of mobile phase aliquots after the second washing cycle with 80% ACN/0.1% TFA or 100% ACN (Fig. 2A) confirmed this phenomenon.

The self-aggregation is known to be closely linked with adsorption processes [40, 41]. The adhesive tryptic tau fragment 299-HVPGGGSVQIVYKPVDLSK-317 contains the hexapeptide 306-VQIVYK-311 established as a minimal interaction motif of PHFs (called PHF6) with a predicted β conformation [6, 35, 42, 43]. It was demonstrated that PHF6 sequence has a high ability to fibrillize in the presence as well as in the absence of added inducers *in vitro* [44]. Subsequently, the peptides 309-VYK-311 and 308-IVYK-311 of the C-termini end of PHF6 sequence were considered as the shortest potential nucleation sites forming PHFs [45, 46]. For detecting amyloid structures characterized by β-sheet formation the ThS assay was performed [47]. The assembly of molecules of synthetic peptide analogue in the presence of heparin in working solutions was confirmed (Fig. 3). These results correspond to the previous papers documenting the assembly of longer peptides containing the PHF6 sequence in the presence of an inducer [44, 48]. A modest assembly rate in the absence of inducer was also shown (except non-buffered acidic solutions and solutions with increased ionic strength) (Fig. 3). It is assumed that aggregated structures/clusters presented in solution can affect the adsorption rate by unspecific depositions of fragment molecules arising from these aggregates/clusters on the solid surface [30, 40]. The data obtained from ThS assay indicate a possible mechanism how the assembly properties may contribute to the nonspecific adsorption in solutions used in the epitope extraction procedure.

Although the results of previous tests confirmed the undesirable properties of the adhesive peptide, we have found a procedure how to eliminate the adhesivity of peptide with aggregation motif and identify the main immunogenic structure of studied protein. The strategy using immobilized trypsin and α-chymotrypsin together proved to be very effective since α-chymotrypsin can cleave the peptide bound 310-YK-311 within the critical PHF6 motif of a tryptic fragment 299-HVPGGGSVQIVYKPVDLSK-317. The significant reduction of adsorption properties of newly formed chymotryptic fragments was observed (Fig. 1C, Supporting information Fig. SI3C).

5 Conclusions

This study shows pitfalls of epitope extraction technique with tau protein, a biomarker of Alzheimer´s disease. We provided information about the adhesive character of the tryptic tau fragment 299-HVPGGGSVQIVYKPVDLSK-317 containing PHF6 minimal aggregation motif and the most hydrophobic tau region. We did not prove the direct relationship of adsorption properties with characteristics of the fragment mentioned above, but even though it seems that these

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

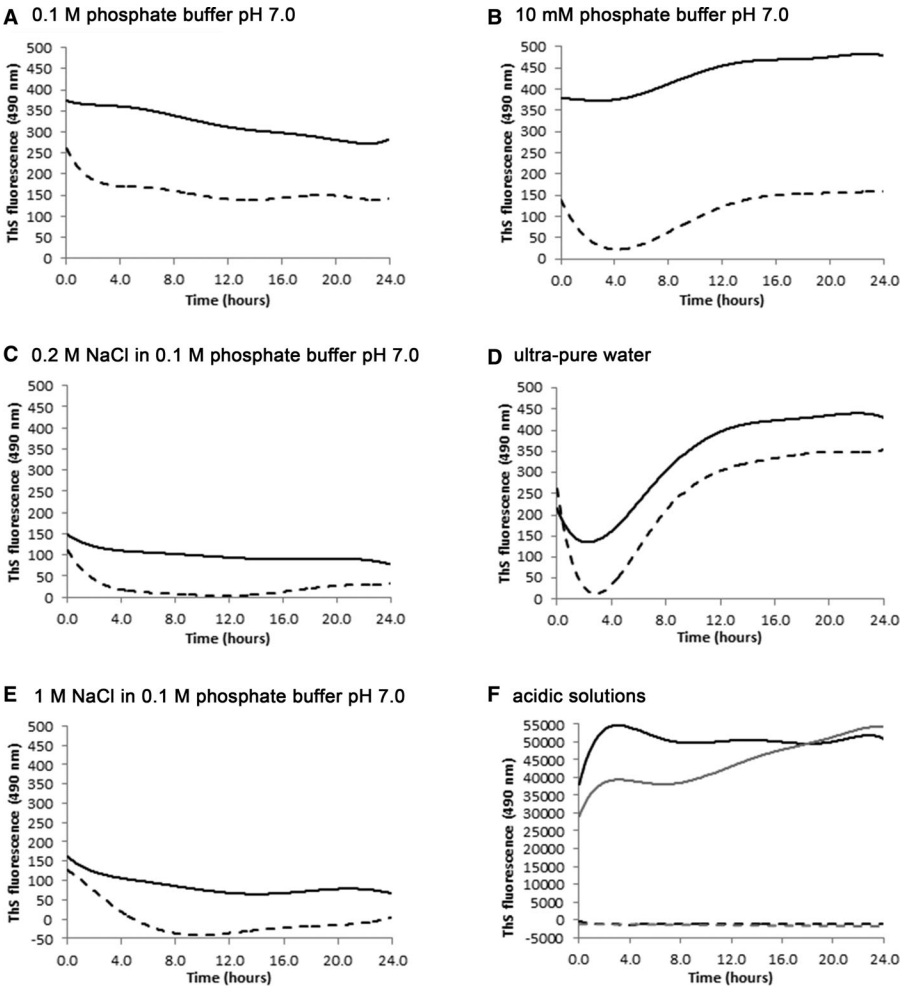


Figure 3. Thioflavin S assay: the curves represent the assembly of synthetic peptide 299-HVPGGGSVQIVYKPVDLSK-317 in the presence (solid lines) or absence (dashed lines) of heparin as an inducer in different solutions (used in epitope extraction protocol and RP-LC). In F) acidic solutions: the black lines represent 0.05% TFA and the gray lines 25% ACN/0.1% TFA. The measurement was carried out in triplets and contained blanks (solution environment contained no tau fragment). The mean values represented in the graph are after subtraction of blank mean values.

characteristics might be associated with significant risk to complicate the structural analysis of tau protein and give results leading to the erroneous interpretation. Our findings show the significance of negative controls and offer the solution how to overcome the difficulties associated with the adhesiveness of peptides linked with specific sequence motifs effectively.

Theoretically estimated peptide fragments of tau protein (human tau-F isoform) including aggregation PHF6 motif and generated by *in-silico* enzymatic digestion with trypsin and with subsequent digestion of the tryptic fragment by α-chymotrypsin (C-term to F/Y/W/M/L, not before P). The amino acid sequence of the PHF6 motif is highlighted in bold.

This work was supported by the research project GACR P304/12/G069, by the project „National Institute of Mental Health (NIMH-CZ)", grant number CZ.1.05/2.1.00/03.0078 and the European Regional Development Fund, by EU project NADINE (No. 246513), and by the Ministry of Education, Youth and Sports of the Czech Republic (Project CZ.1.07/2.3.00/30.0021 "Enhancement of R&D Pools of Excellence at the University of Pardubice").

The authors have declared no conflict of interest.

6 References

[1] Carrell, R. W., Lomas, D. A., *Lancet* 1997, *350*, 134–138.

[2] Golde, T. E., Borchelt, D. R., Giasson, B. I., Lewis, J., *J. Clin. Invest.* 2013, *123*, 1847–1855.

[3] Jucker, M., Walker, L. C., *Ann. Neurol.* 2011, *70*, 532–540.

[4] Lee, V. M., Goedert, M., Trojanowski, J. Q., *Annu. Rev. Neurosci.* 2001, *24*, 1121–1159.

[5] Gamblin, T. C., *Biochim. Biophys. Acta* 2005, *1739*, 140–149.

[6] Li, W., Lee, V. M.-Y., *Biochemistry (Mosc.)* 2006, *45*, 15692–15701.

[7] Selenica, M.-L. B., Davtyan, H., Housley, S. B., Blair, L. J., Gillies, A., Nordhues, B. A., Zhang, B., Liu, J., Gestwicki, J. E., Lee, D. C., Gordon, M. N., Morgan, D., Dickey, C. A., *J. Neuroinflamm.* 2014, *11*, 152–163.

[8] Khuebachova, M., Verzillo, V., Skrabana, R., Ovecka, M., Vaccaro, P., Panni, S., Bradbury, A., Novak, M., *J. Immunol. Methods* 2002, *262*, 205–215.

[9] Smith, G. P., *Science* 1985, *228*, 1315–1317.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[10] Cho, W., Jung, K., Regnier, F. E., *J. Sep. Sci.* 2010, *33*, 1438–1447.

[11] McLaurin, J., Cecal, R., Kierstead, M. E., Tian, X., Phinney, A. L., Manea, M., French, J. E., Lambermon, M. H. L., Darabie, A. A., Brown, M. E., Janus, C., Chishti, M. A., Horne, P., Westaway, D., Fraser, P. E., Mount, H. T. J., Przybylski, M., St George-Hyslop, P., *Nat. Med.* 2002, *8*, 1263–1269.

[12] Paraschiv, G., Vincke, C., Czaplewska, P., Manea, M., Muyldermans, S., Przybylski, M., *J. Mol. Recognit.* 2013, *26*, 1–9.

[13] Hager-Braun, C., Tomer, K. B., *Expert Rev. Proteomics* 2005, *2*, 745–56.

[14] Jeyarajah, S., Parker, C. E., Sumner, M. T., Tomer, K. B., *J. Am. Soc. Mass Spectrom.* 1998, *9*, 157–165.

[15] Jankovicova, B., Rosnerova, S., Slovakova, M., Zverinova, Z., Hubalek, M., Hernychova, L., Rehulka, P., Viovy, J.-L., Bilkova, Z., *J. Chromatogr. A* 2008, *1206*, 64–71.

[16] Skultety, L., Jankovicova, B., Svobodova, Z., Mader, P., Rezacova, P., Dubrovcakova, M., Lakota, J., Bilkova, Z., *J. Proteome Res.* 2010, *9*, 5171–5179.

[17] Jankovicova, B., Skultety, L., Dubrovcakova, M., Stern, M., Bilkova, Z., Lakota, J., *Immunol. Lett.* 2013, *153*, 47–49.

[18] Bílková, Z., Stefanescu, R., Cecal, R., Korecká, L., Ouzká, S., Jezová, J., Viovy, J.-L., Przybylski, M., *Eur. J. Mass Spectrom.* 2005, *11*, 489–495.

[19] Bohoyo, D., Le Potier, I., Rivière, C., Klafki, H., Wiltfang, J., Taverna, M., *J. Sep. Sci.* 2010, *33*, 1090–1098.

[20] Skrabana, R., Sevcik, J., Novak, M., *Cell. Mol. Neurobiol.* 2006, *26*, 1085–1097.

[21] Staros, J. V., *Biochemistry (Mosc.)* 1982, *21*, 3950–3955.

[22] Erlanger, B. F., Kokowsky, N., Cohen, W., *Arch. Biochem. Biophys.* 1961, *95*, 271–278.

[23] Bílková, Z., Mazurová, J., Churácek, J., Horák, D., Turková, J., *J. Chromatogr. A* 1999, *852*, 141–149.

[24] Herbert, B., Galvani, M., Hamdan, M., Olivieri, E., MacCarthy, J., Pedersen, S., Righetti, P. G., *Electrophoresis* 2001, *22*, 2046–2057.

[25] Schägger, H., *Nat. Protoc.* 2006, *1*, 16–22.

[26] Hermanson, G. T., *Bioconjugate Techniques*, Academic Press, New York 2013, pp. 227–228.

[27] Kucerova, J., Svobodova, Z., Knotek, P., Palarcik, J., Vlcek, M., Kincl, M., Horak, D., Autebert, J., Viovy, J.-L., Bilkova, Z., *Mater. Sci. Eng. C* 2014, *40*, 308–315.

[28] Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., Roepstorff, P., *J. Mass Spectrom.* 1999, *34*, 105–116.

[29] Franc, V., Řehulka, P., Medda, R., Padiglia, A., Floris, G., Šebela, M., *Electrophoresis* 2013, *34*, 2357–2367.

[30] Rabe, M., Verdes, D., Seeger, S., *Adv. Colloid Interface Sci.* 2011, *162*, 87–106.

[31] Friedhoff, P., Schneider, A., Mandelkow, E. M., Mandelkow, E., *Biochemistry (Mosc.)* 1998, *37*, 10223–10230.

[32] Becker, J. S., Zoriy, M., Przybylski, M., Becker, J. S., *Int. J. Mass Spectrom.* 2007, *261*, 68–73.

[33] Becker, J. S., Przybylski, M., *J. Anal. At. Spectrom.* 2007, *22*, 761–765.

[34] Jeyachandran, Y. L., Mielczarski, J. A., Mielczarski, E., Rai, B., *J. Colloid Interface Sci.* 2010, *341*, 136–142.

[35] von Bergen, M., Barghorn, S., Biernat, J., Mandelkow, E.-M., Mandelkow, E., *Biochim. Biophys. Acta - Mol. Basis Dis.* 2005, *1739*, 158–166.

[36] Mukrasch, M. D., Bibow, S., Korukottu, J., Jeganathan, S., Biernat, J., Griesinger, C., Mandelkow, E., Zweckstetter, M., *PLoS Biol.* 2009, *7*, 399–414.

[37] Jones, E. M., Dubey, M., Camp, P. J., Vernon, B. C., Biernat, J., Mandelkow, E., Majewski, J., Chi, E. Y., *Biochemistry (Mosc.)* 2012, *51*, 2539–2550.

[38] Perret-Liaudet, A., Pelpel, M., Tholance, Y., Dumont, B., Vanderstichele, H., Zorzi, W., Elmoualij, B., Schraen, S., Moreaud, O., Gabelle, A., Thouvenot, E., Thomas-Anterion, C., Touchon, J., Krolak-Salmon, P., Kovacs, G. G., Coudreuse, A., Quadrio, I., Lehmann, S., *J. Alzheimers Dis. JAD* 2012, *31*, 13–20.

[39] Fekete, S., Beck, A., Wagner, E., Vuignier, K., Guillarme, D., *J. Sep. Sci.* 2015, *38*, 1–8.

[40] Rabe, M., Verdes, D., Seeger, S., *Soft Matter* 2009, *5*, 1039–1047.

[41] Zhang, S., Shao, T., Bekaroglu, S. S. K., Karanfil, T., *Environ. Sci. Technol.* 2009, *43*, 5719–5725.

[42] von Bergen, M., Friedhoff, P., Biernat, J., Heberle, J., Mandelkow, E. M., Mandelkow, E., *Proc. Natl. Acad. Sci. U S A* 2000, *97*, 5129–5134.

[43] Eliezer, D., Barré, P., Kobaslija, M., Chan, D., Li, X., Heend, L., *Biochemistry (Mosc.)* 2005, *44*, 1026–1036.

[44] Santa-María, I., Pérez, M., Hernández, F., Muñoz, V., Moreno, F. J., Avila, J., *Biochim. Biophys. Acta - Mol. Basis Dis.* 2006, *1762*, 683–692.

[45] Goux, W. J., Kopplin, L., Nguyen, A. D., Leak, K., Rutkofsky, M., Shanmuganandam, V. D., Sharma, D., Inouye, H., Kirschner, D. A., *J. Biol. Chem.* 2004, *279*, 26868–26875.

[46] Rojas Quijano, F. A., Morrow, D., Wise, B. M., Brancia, F. L., Goux, W. J., *Biochemistry (Mosc.)* 2006, *45*, 4638–4652.

[47] Chirita, C. N., Congdon, E. E., Yin, H., Kuret, J., *Biochemistry (Mosc.)* 2005, *44*, 5862–5872.

[48] Mizushima, F., Minoura, K., Tomoo, K., Sumida, M., Taniguchi, T., Ishida, T., *J. Biochem. (Tokyo)* 2007, *142*, 49–54.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Publication II:

Identification and characterization of natural antibodies against tau protein in an intravenous immunoglobulin product.

Hromadkova, L., Kolarova, M., Jankovicova, B., Bartos, A., Ricny, J., Bilkova, Z., Ripova, D., Journal of Neuroimmunology, 2015, 289, 121-129.

Stated contributions of the author: Study design. Experimental part (SDS-PAGE electrophoresis, tau phosphorylation, blotting techniques, avidity dot-blot immunoassay). Data analysis. Manuscript design and writing.

Journal of Neuroimmunology 289 (2015) 121–129

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Identification and characterization of natural antibodies against tau protein in an intravenous immunoglobulin product

Lenka Hromadkova [a,b,c,1], Michala Kolarova [a,d,*,1], Barbora Jankovicova [c], Ales Bartos [a,d], Jan Ricny [a], Zuzana Bilkova [c], Daniela Ripova [a]

[a] Department of Neurobiology, AD Center, National Institute of Mental Health, Klecany, Czech Republic
[b] Faculty of Science, Charles University in Prague, Prague, Czech Republic
[c] Department of Biological and Biochemical Sciences, University of Pardubice, Pardubice, Czech Republic
[d] Third Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form 21 October 2015
Accepted 24 October 2015

Keywords:
Alzheimer disease
Avidity
Immunoreactivity
Intravenous immunoglobulins
Natural antibodies
Tau protein

ABSTRACT

The latest therapeutic approaches to Alzheimer disease are using intravenous immunoglobulin (IVIG) products. Therefore, the detailed characterization of target-specific antibodies naturally occurring in IVIG products is beneficial. We have focused on characterization of antibodies isolated against tau protein, a biomarker of Alzheimer's disease, from Flebogamma IVIG product. The analysis of IgG subclass distribution indicated skewing toward IgG3 in anti-tau-enriched IgG fraction. The evaluation of their reactivity and avidity with several recombinant tau forms was performed by ELISA and blotting techniques. Truncated non-phosphorylated tau protein (amino acids 155–421) demonstrated the highest reactivity and avidity index. We provide the first detailed insight into the reactivity of isolated natural antibodies against tau protein.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The concept that humoral immunity may play a particular role during the development and progression of Alzheimer disease (AD) has an effect on trends in AD therapeutic strategies. Especially, the antibody-based immunotherapy focused on structurally modified forms of amyloid peptide and tau protein has gained a greater importance mainly since AD is considered as a protein conformational disorder (Carrell and Lomas, 1997; Soto, 1999). The fact that numerous natural antibodies reactive with different neuronal proteins have been proved both in AD and control serum increases general interest in revealing their relevance and therapeutic potential (Bahmanyar et al., 1983; Bartos et al., 2012; Levin et al., 2010; Rosenmann et al., 2006; Terryberry et al., 1998; Watts et al., 1981).

As a relatively new encouraging strategy for AD, therapy proposed the use of intravenous immunoglobulin products (IVIG) prepared from plasma of healthy donors, which represent a reservoir of natural autoantibodies comprising approximately two-thirds of the human immune repertoire of IgG (Pul et al., 2011). A notable property of most natural antibodies is their polyreactivity (polyspecificity) and the associated variable, predominantly low, antigen-binding affinities (Avrameas and Ternynck, 1993; Jianping et al., 2006; Sedykh et al., 2013; Szabo et al., 2010). These polyclonal antibodies recognize multiple epitopes, and more likely have stronger therapeutic effects than the passive immunization with monoclonal antibodies (Kayed et al., 2011). Another fact supporting IVIG products in AD therapy originates from their potent immunomodulatory and anti-inflammatory effect beneficial in many diseases, e.g. immune deficiency, autoimmune to cancer diseases, and even in a number of immune-mediated neurological disorders (Fuchs et al., 2008; Kajii et al., 2014; Seite et al., 2008; Stangel and Pul, 2006). Numerous studies with various results of natural anti-Aβ antibody levels in IVIG have been published (Balakrishnan et al., 2010; Dodel et al., 2002; Du et al., 2003; Klaver et al., 2010a,b, 2013; Szabo et al., 2008).

In recent years, interest in tau protein as a therapeutic target in AD has increased. This trend is supported by facts that tau pathology better correlates with AD progression and that it may be an independent process from Aβ accumulation (Arriagada et al., 1992a, 1992b; Braak et al., 1999; Gomez-Isla et al., 1997). Two papers about natural anti-tau antibodies in IVIG are currently known (Smith et al., 2013, 2014). They have proved that three IVIG products exhibit different concentrations of anti-tau specific antibodies and even high variability in percentage of antibodies against different tau fragments relative to total levels of

* Corresponding author at: Department of Neurobiology, AD Center, National Institute of Mental Health, Klecany, Czech Republic.
E-mail address: michala.kolarova@nudz.cz (M. Kolarova).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jneuroim.2015.10.017
0165-5728/© 2015 Elsevier B.V. All rights reserved.

anti-tau antibody contained in these products (Smith et al., 2013, 2014). It is reasonable to characterize the target-reactive antibodies in IVIG products whether their anticipated therapeutic effect could be significantly influenced by their concentration or even by the abundance of antibodies preferentially binding with particular epitopes of pathological protein forms.

In this study, we focused on the characterization of isolated natural antibodies against tau protein (nTau-Abs) from IVIG product Flebogamma DIF (5 g/100 ml, Grifols Biologicals Inc., Los Angeles, CA, USA). We isolated antibodies against human full-length form of tau protein by low-pressure affinity chromatography. The IgG subclass distribution was assessed. Subsequently, we compared the reactivity of all fractions of the isolation procedure with different non-phosphorylated/phosphorylated human full-length and truncated tau forms by ELISA. Blotting techniques were applied to assess the reactivity of isolated nTau-Abs in comparison with two anti-tau antibodies; monoclonal tau46.1 and polyclonal rabbit anti-tau antibodies. Moreover, the avidity index of isolated antibodies was measured against full-length and truncated tau forms by using chaotropic reagent in ELISA and dot-blot assays. We observed the highest reactivity and avidity index of isolated antibodies with the truncated non-phosphorylated form, tau 155–421. Our study can partly contribute to clarify the natural antibody diversity in pooled, highly concentrated immunoglobulin product Flebogamma, which is now examined in ongoing phase III trial (Grifols Biologicals Inc., 2015).

2. Materials and methods

2.1. Materials

cDNA of recombinant human tau proteins and tau monoclonal antibody (tau-46.1) were a generous gift from Dr. Francisco Garcia-Sierra (Mexico City, Mexico) and Dr. Lester I. Binder (Chicago, USA). Recombinant human tau protein (isoform 2N4R) was purchased from rPeptide (Bogart, GA, USA). *Escherichia coli* strain BL21 (DE3) (*E. coli* B F− dcm ompT hsdS(rB− mB−) gal λ(DE3) comes from Stratagene (San Diego, CA, USA). The IVIG product Immune Globulin Intravenous (Human) Flebogamma DIF (5 g/100 ml) was ordered from Grifols Biologicals Inc. (Los Angeles, CA, USA). Recombinant protein kinase ERK2 (401 000 U/mg) and mouse cAMP-dependent protein kinase (catalytic subunit Cα, PKA, >15 U/mg) were ordered from Biaffin GmbH & Co KG (Kassel, Germany). Glycogen Synthase Kinase 3 (GSK-3, 500 000 U/ml) and adenosine 5′-triphosphate (ATP, 10 mM) were acquired from New England Biolabs (Ipswich, MA, USA). Monoclonal mouse phospho-PHF-tau pSer202/Thr205 antibody (AT8) and phospho-PHF-tau pThr231 antibody (AT180) were purchased from Thermo Scientific (Waltham, MA, USA) and polyclonal rabbit anti-tau phospho-Ser396 from GenScript (Piscataway, NJ, USA). F(ab′)2-goat anti-human IgG (Fc specific, highly cross adsorbed/HRP conjugate) was purchased from Novex, Life Technologies (Carlsbad, CA, USA) and goat anti-rabbit IgG (HRP conjugate) from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl-β-thiogalactopyranosid (IPTG), bovine serum albumin (BSA; 98% electrophoresis), tetramethylbenzidine (TMB; 98% TLC), DL-dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA) and NH_4SCN (p.a.) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PVDF membrane (Immuno-Blot PVDF Membrane, 0.2 μm), nitrocellulose membrane (0.2 μm), Precision Plus Protein™ WesternC™ Standard as a molecular marker, Clarity Western ECL substrate and Immun-Blot Opti-4CN Colorimetric kit were acquired from Bio-Rad (Hercules, CA, USA). BCA protein assay kit was obtained from Thermo Scientific (Waltham, MA, USA). All other chemicals were of reagent grade. The IgG Subclass ELISA Kit was obtained from Invitrogen (Camarillo, CA, US).

Resin Labiomer 300 was ordered from Labio (Prague, Czech Republic), and Ni Sepharose 6 Fast Flow resin was acquired from GE Healthcare (Wilmington, MA, USA). Empty Econo-Pac Chromatography columns and Trans-Blot® SD Semi-Dry Transfer Cell were obtained from Bio-Rad (Hercules, CA, USA). Amicon® Ultra 15 ml filters 30K (30 kDa molecular weight cut-off) were purchased from Merck Millipore (Billerica, MA, USA). 96-well microplates (Nunc Immunoplate F96 Maxisorp) were provided by Nunc, Thermo Scientific (Waltham, MA, USA). Dot-blot DHM-96 unit manifold was purchased from Scie-Plast (Cambridge, UK). Spectrophotometer Eppendorf 6131 was from Eppendorf (Hamburg, Germany).

2.2. Methods

2.2.1. Isolation of naturally occurring antibodies against tau protein from IVIG

We purified antibodies against tau protein from Flebogamma IVIG product by low-pressure affinity chromatography. The column was prepared as follows: 25 mg of His-tagged tau 1–441 protein was reacted with 5 ml of pre-packed resin (Labiomer 300, epoxy-activated, 50 μm bead size in Econo-Pac column of 14 cm length and 1.5 cm diameter), residual reactive epoxide groups were blocked with 0.2 M ethanolamine overnight at 4 °C and then the column was equilibrated with PBS buffer (Hermanson, 2013). IVIG (8 ml, Flebogamma) were loaded on a column by flow 0.1 ml/min and flow-through fraction in an amount of 15 ml was collected. The column was washed with 45 ml PBS buffer and immunocaptured Abs were eluted by 10.5 ml 0.1 M glycine-HCl buffer pH 2.6. Eluted fraction was immediately neutralized by 1 M Tris-base until pH 8–9. Purified Abs were then concentrated in centrifugal filter units (Amicon-Ultra 30K) to 2 ml final volume and stored in PBS pH 7.2 with 50% glycerol at protein concentration 0.8 mg/ml at −20 °C. The IgG amount in initial IVIG fraction, flow-through fraction and concentrated isolated fraction (prior to the addition of glycerol) was spectrophotometrically determined at absorbance 280 nm with parallel to PBS buffer as a blank. Percentages of IgG subclasses were assayed using an IgG Subclass ELISA Kit, according to the manufacturer's instructions, for initial IVIG and isolated antibody samples.

2.2.2. Reactivity evaluation of isolated antibodies with tau proteins

We tested the reactivity of isolated natural antibodies with several tau forms (their preparation in Supplemental materials) by two blotting immunoassays, Western blot and dot-blot. Western blot was carried out to confirm the reactivity with both unphosphorylated and phosphorylated tau forms. Whereas the dot-blot immunoassay was carried out with only unphosphorylated recombinant tau forms due to reactivity comparison with rabbit anti-tau antibodies isolated from immunized serum.

2.2.2.1. Western blot immunoassay. Unphosphorylated and phosphorylated tau samples (Suppl): tau 1–441 rPeptide, His-tagged tau 1–441, and truncated His-tagged forms: tau 155–421 and tau 13–391 (5 μg of each) mixed with reducing sample buffer in the volume ratio 1:1, were loaded into Tricine polyacrylamide gel (10% T, 3% C) and then transferred onto nitrocellulose membrane. The membrane was blocked by 5% defatted milk in PBS-0.1% Tween 20 (PBS-T) for 1 h at RT. Isolated anti-tau antibodies (1:250) were added in PBS-T with 1% BSA and incubated with the membrane overnight at 4 °C. The unbound IgG molecules were removed by PBS-T washing five times (each 5 min). The membrane was incubated with goat anti-human IgG antibody HRP-conjugate at 1:15 000 dilution in PBS-T with 1% BSA for 2 h at RT and subsequently washed by PBS-T five times (each 5 min). The chemiluminescence detection by Clarity western ECL substrate according to the manufacturer's instructions followed. ChemiDoc™ XRS + Imaging System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA) was applied for documentation.

2.2.2.2. Dot blot immunoassay. Dot-blot analysis was performed with colorimetric 4-CN detection that is more suitable for subsequently used avidity dot-blot immunoassay. Additionally, for comparison purpose

we applied in this experiment our rabbit polyclonal anti-tau antibody raised against His-tagged tau 1–441 protein form (Kristofikova et al., 2014), and purified by low-pressure affinity chromatography using tau 1–441 rPeptide without His-tag. Three different unphosphorylated recombinant tau proteins listed in Suppl and tau 1–441 rPeptide (for each: 1 μg and 0.5 μg/100 μl 0.1 M PBS pH 7.0) were spotted onto PVDF membrane using a Dot-blot DHM-96 unit manifold and incubated in Opti-4CN kit blocking solution in PBS-T for 1 h at RT. Anti-tau antibodies isolated from IVIG (1:250) or immunized rabbit serum (1:250) were added in PBS-T with 1% BSA and incubated with the membrane for 1 h at RT. The unbound IgG molecules were removed by PBS-T washing three times. The membrane was incubated with goat anti-human IgG antibody HRP-conjugate/goat anti-rabbit antibody HRP-conjugate (1:1000) in PBS-T with 1% BSA for 1 h at RT and subsequently washed by PBS-T three times. The spots were visualized by Opti-4CN kit according to the manufacturer's instructions. ChemiDoc™ XRS + Imaging System with Image Lab™ Software was applied for documentation and spot density analysis.

2.2.3. Quantification of anti-tau natural antibodies by ELISA immunoassay

Levels of antibodies against four forms of tau protein (tau 1–441 rPeptide, His-tagged tau 1–441 and truncated tau 155–421 and tau 13–391 protein forms) in both unphosphorylated/ phosphorylated states were measured in initial IVIG fraction, flow-through fraction and eluted fraction by ELISA. All samples were measured in duplicates. Tau antigen in 0.1 M carbonate buffer pH 9.5 was applied to microplate (0.2 μg/50 μl per well) and incubated overnight at 4 °C. Then the wells were blocked by 1% BSA in PBS-T for 1 h at room temperature (RT). Subsequently, the microplate was washed 3 times by 0.1% BSA in PBS-T. Initial IVIG fraction, flow-through fraction and eluted fraction were diluted as follows 1:100, 1:300, 1:900, 1:2700, 1:8100, 1:24 300, 1:72 900 by 1% BSA in PBS-T. Diluted antibodies (0.1 ml/well) were added into the plate and incubated 2 h at RT. The unbound molecules were removed by five times washing with 0.1% BSA in PBS-T. Incubation with 0.1 ml/well of goat anti-human IgG antibody HRP-conjugate at dilution 1:10 000 for 30 min at RT followed and then the microplate was washed five times as above. Final incubation with 0.1 ml/well of TMB substrate was performed for 30 min at RT in the dark. The reaction was stopped by 0.1 ml of 1 M H_2SO_4 per well, and the absorbance was measured by Elisa Reader Multiskan EX (Thermo Scientific) at 450 nm and 620 nm as a reference wavelength.

2.2.4. Measurement of avidity index

The avidity index of isolated anti-tau antibodies from IVIG product as well as from immunized rabbit serum was determined by chaotropic agent ammonium thiocyanate (NH_4SCN) in two independent methods, ELISA and dot-blot immunoassay. The experimental conditions as in Sections 2.2.2.2 and 2.2.3 were retained with extra incubation step. In ELISA experiment, after washing of unbound anti-tau antibody molecules which were applied in dilution 1:250 against recombinant tau proteins (0.1 μg/well), additional 10 min incubation with NH_4SCN in PBS pH 7.0 within molarity range 0–2.1 M was included (Pullen et al., 1986). In avidity dot-blot immunoassay, for avidity evaluation of isolated anti-tau antibodies (dilution 1:200) against recombinant tau proteins (1 μg of tau 155–421 His-tag, 5 μg of tau 1–441 His-tag or tau 13–391 His-tag), additional 5 min incubation step with NH_4SCN in PBS pH 7.0 within molarity range 0–2.1 M was included after washing of unbound IgG molecules (Svobodova et al., 2013). All samples were measured in duplicates for ELISA and in triplicates for dot blot analysis. Avidity index is defined as molar concentration of thiocyanate solution (M) that causes the decrease of initial sample signal (incubation in PBS buffer pH 7.0 without NH_4SCN) on the value of 50% in ELISA and dot-blot avidity immunoassay. The thiocyanate molarity corresponding to 50% reduction in the initial sample signal was calculated from the linear regression equation of logarithmic transformed values of % signal intensity relative to initial signal for each thiocyanate molar concentration (i.e., log (signal / initial signal) × 100).

2.2.5. Statistics

Data were analyzed with GraphPad Prism software Version 5.0 from GraphPad (San Diego, CA, USA). For comparison of antibody levels against various tau forms in all fractions collected within the purification procedure we chose values at absorbance 0.5 as these lie in linear range of the curve in ELISA measurements ($Y = Bmax * X / (Kd + X) + C$). Student t-test was used for analysis of specific IgG levels among initial IVIG fraction (Flebogamma), flow-through fraction and elution fraction for all used tau antigens. A p-value < 0.01 was considered statistically significant.

3. Results

3.1. Purification of natural antibodies with specificity against tau protein

In previously published papers, the Flebogamma IVIG product characterization revealed that IgG content is more than 99 % with the percentage of individual IgG subclasses similar to normal serum values, IgM and IgA are present in only trace amounts (Ballow, 2009; Jorquera, 2009). Based on these data, we expected that the isolated antibodies belong to IgG class at least in 99% purity. We obtained approximately 3.2 mg IgG molecules by acidic elution which represents 0.8 % from 400 mg of total IgG amount contained in IVIG fraction applied to the column and transferred them into PBS buffer by Amicon 30K filters. Subsequently, we determined the IgG subclass distribution in the initial IVIG product and the fraction containing antibodies isolated against tau protein (elution fraction) by ELISA kit. IgG1 constitutes the major subclass proportion in the two samples, 61.7% and 57.6% respectively, and IgG4 represents the least abundant IgG subclass, 1.9% and 1.7% respectively. A fourfold increase in IgG3 proportion (4.3% to 16%) and slight decrease of IgG2 amount (32.1% to 24.8%) were observed in the elution fraction in comparison to the initial IVIG product (Fig. 1A, B). The purity and integrity of IgG molecules were verified by non-reducing SDS-PAGE electrophoresis (Fig. 2). The molecular weight ranges from 150 kDa to more than 250 kDa which may be attributed to anomalous behavior of IgG during SDS-PAGE (Fasler et al., 1988) and to dimers occurrence in IVIG (Miescher et al., 2005; Vassilev et al., 1995; Wymann et al., 2008). By densitometric analysis with ImageLab 4.0 software, IgG fragments or contaminants in elution fraction corresponding to molecular weights of 92 kDa and 53.5 kDa were determined below 1% from the line total density (Fig. 2 — position 3).

3.2. Reactivity evaluation of purified antibodies by blotting techniques

The reactivity of isolated nTau-Abs with unphosphorylated and phosphorylated forms of four tau proteins (tau 1–441 rPeptide and three His-tagged forms: tau 1–441, tau 13–391, and tau 155–421, see Supplemental materials) was evaluated by Western blot analysis (Fig. 3). The results revealed that the reactivity of isolated nTau-Abs with the phosphorylated form of His-tagged tau 1–441 was significantly decreased comparing with its non-phosphorylated form (Fig. 3 — positions 3, 4). Therefore, the sufficiency of electrotransfer of all full-length tau antigens was verified by reprobing the same nitrocellulose membrane with mAb tau 46.1 (epitope 428–441; (Garcia-Sierra et al., 2003). The Western blot analysis using nTau-Abs also provided us with additional information about applied tau molecules. In addition to information about molecular weights with respect to fragmentation level and distribution of monomers, dimers and higher molecular structures, the shift in molecular weight caused by posttranslational modifications, herein by phosphorylation, is also illustrated. Moreover, we can see that the truncated tau protein molecules were more prone to aggregation also under the reducing conditions (Fig. 3 — positions 5–8). This is probably caused by conformational changes of the fragments due to the phosphorylation and truncation at Asp421 or Glu391 which

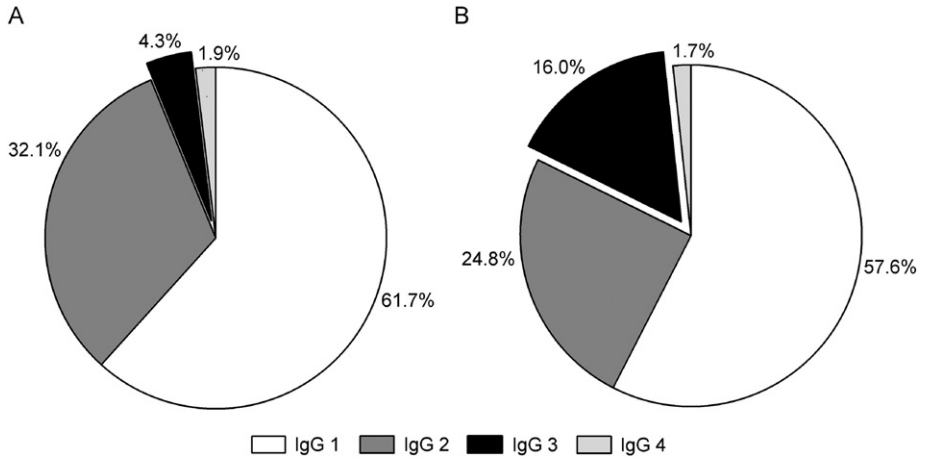


Fig. 1. The IgG subclass distribution in the initial IVIG product (A) and the fraction of antibodies isolated against tau protein (B). IgG subclass percentages were determined by ELISA kit. The IgG1 and IgG4 distribution is comparable in both samples. The percentage of IgG3 increases four times and of IgG2 slightly decreases in isolated antibody fraction in comparison to the initial IVIG fraction.

facilitates assembly into aggregates (Ding et al., 2006; Gamblin et al., 2003; Garcia-Sierra et al., 2008; Guillozet-Bongaarts et al., 2005; Hanger and Wray, 2010; Kolarova et al., 2012).

Dot-blot immunoassay was carried out with two anti-tau antibodies; human natural anti-tau antibodies isolated from IVIG and rabbit anti-tau antibodies isolated from serum immunized with His-tagged tau 1–441. Despite the fact that nTau-Abs from IVIG were purified using the His-tagged tau 1–441, the spot signal was the weakest with this antigen form (Fig. 4, A — position 2) in comparison with His-tagged truncated tau forms (tau 13–391 and tau 155–421) (Fig. 4, A — positions 3, 4). Tau 1–441 rPeptide (Fig. 4, A — position 1) was used as control antigen to demonstrate that tag composed of 6 histidines is not the immunodominant epitope and has a minimal effect on enhancement of immunoreactivity in this case. This corresponds with our conviction that His-tag structure is not naturally occurring antigenic motif in the human body, and thus the epitopes reactive with nTau-Abs are located within tau molecule sequence. Moreover, this idea is supported by the reactivity of polyclonal anti-tau antibody from rabbit immunized by His-tagged tau 1–441 and purified by tau 1–441 rPeptide which provides the highest signal with immunogenic tau 1–441 containing His-tag irrespective of the ligand used in affinity chromatography, tau 1–441 rPeptide (Fig. 4, B — positions 1, 2).

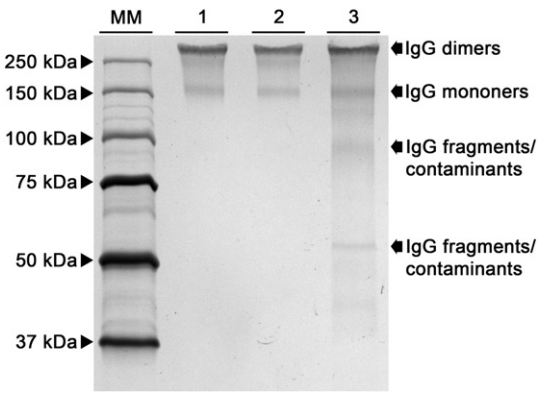


Fig. 2. SDS-PAGE electrophoresis of initial IVIG fraction (1), a flow-through fraction (2) and elution fraction containing natural antibodies in PBS pH 7.2 with 50% glycerol (3) followed by silver staining. Molecular marker 10–250 kDa (MM) was applied to determine the molecular weight of isolated IgG. Arrowed bands represent >250 kDa band of IgG dimers, 149.2 kDa band of IgG monomers. Bands of 92 kDa and 53.5 kDa molecular weight are supposed to be IgG fragments or contaminants representing 1% of total content determined by density analysis (3).

3.3. Evaluation of anti-tau antibodies amount by ELISA

Initial IVIG fraction (Flebogamma, 50 mg IgG/ml; IF), flow-through fraction (IgG passed through the column with covalently bound antigen tau 1–441 His-tag, 7.9 mg IgG/ml; FTF) and elution fraction (affinity purified IgG fraction retained in the column with covalently bound antigen tau 1–441 in His-tagged form, concentrated after the elution by Amicon 30K filter device to final concentration of 0.8 mg IgG/ml; EF) were applied in ELISA. Graphical representation of antibody reactivity with His-tagged tau 1–441 expressed as the relationship between detected signal (absorbance at 450 nm) and the logarithm of antibody dilution was used to evaluate the efficiency of natural anti-tau antibody isolation procedure. As can be seen in Fig. 5 we isolated and concentrated most of the nTau-Abs against human full-length tau protein from IVIG product into elution fraction comparing to minimal signal in flow-through fraction.

Subsequently, we carried out ELISA assay to measure the levels of natural antibodies occurring in IVIG product within the purification procedure. We used His-tagged tau forms (preparation method in Suppl 1.1) and commercial tau 1–441 rPeptide as well as their phosphorylated equivalents (phosphorylation method in Suppl 1.2). The dilution factors of reactive antibodies for each tau form giving OD 0.5 at absorbance 450 nm were calculated according to 2.2.5. The graphical representation allowed us to evaluate the difference in levels of reactive antibodies with particular tau forms among purification steps. Slight differences were apparent in the reactivity of initial IVIG fraction and elution fraction with phosphorylated tau forms caused by tau ligand used in purification procedure. Especially, the isolated antibodies showed much lower dilution to get OD 0.5 for phosphorylated His-tagged tau 155–421 form (Fig. 6A) in contrast to non-phosphorylated form. On the other hand, this fragment was highly antigenic as we can see high reactivity with all fractions and this reactivity was abolished by phosphorylation of this fragment in all fractions, respectively. We also observed a decrease in dilution factor for a flow-through fraction in comparison with initial IVIG and elution fractions for all antigens except His-tagged tau 155–421.

In order to express variation in reactive antibody concentrations among purification fractions, we calculated the amount of reactive IgG molecules (μg/well) based on spectrophotometrically determined concentration in each fraction giving OD 0.5 in ELISA measurements. Then, we were able to observe statistically significant differences in antibody levels at OD 0.5 for various tau forms among particular fractions. Moreover, these data indicated that antibodies interacting with tau proteins are concentrated in elution fraction (Fig. 6B). We found a significant decrease of IgG amounts required to get OD 0.5 in elution fraction

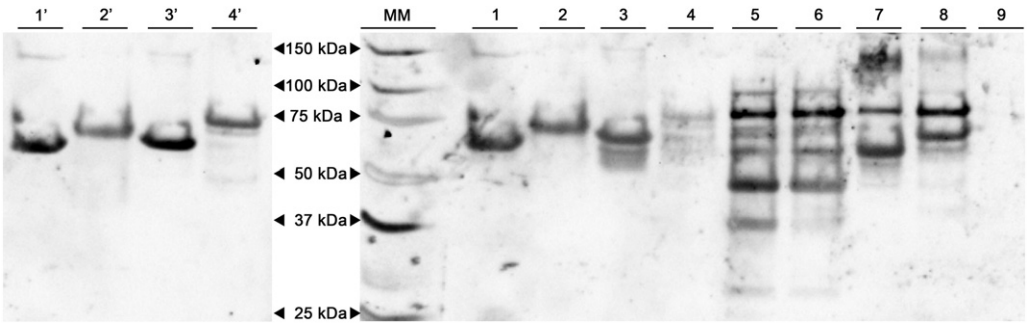


Fig. 3. Western blot analysis performed with isolated natural Abs to evaluate their specific reactivity with tau antigens in non-phosphorylated (np-) and phosphorylated form (p-): np-tau 1–441 rPeptide (1), p-tau 1–441 rPeptide (2) and His-tagged tau forms; np-tau 1–441 (3), p-tau 1–441 (4), np-tau 155–421 (5), p-tau 155–421 (6), np-tau 13–391 (7), p-tau 13–391 (8). The cross-reactivity with soluble kinases from kinase cocktail was disproved by kinases loading in the same ratio and no reactivity with applied Abs was detected (9). After reprobing the nitrocellulose membrane, the full-length tau forms (1, 2, 3, 4) were immunostained with mAb tau-46.1 (epitope 428–441 aa) to verify the sufficiency of electrotransfer (1′, 2′, 3′, 4′).

in contrast to initial IVIG and flow-through fractions. The measured values expressed as both dilutions, and total amounts of natural anti-tau antibodies in elution fraction against all used tau protein forms are summarized in Table 1.

3.4. Avidity index determination of isolated natural anti-tau antibodies

After comparing the levels of nTau-Abs in all sample fractions, we focused on determination of avidity index for isolated IgG molecules against all three His-tagged tau antigens; two truncated tau forms (tau 13–391 and tau 155–421) and tau 1–441 as an antigen through which specific antibodies were purified. In the graphical representation, the signal intensity [%] of antibodies represents the percentage decrease in the binding as a function of the thiocyanate molar concentration (Fig. 7). The calculation of avidity index from the log-transformed curve is mentioned in 2.2.6. Avidity index of nTau-Abs with His-tagged tau 155–421 gave the greatest values and did not significantly vary between dot blot and ELISA techniques, particularly 1.67 M/1.61 M of NH_4SCN. All avidity indexes for isolated natural anti-tau antibodies are summarized in Table 1. The decrease in signal intensity with increasing NH_4SCN molarity is presented in a graphic form for both methods; ELISA and avidity dot-blot immunoassay (Fig. 7A, B). Avidity dot-blot immunoassay was also carried out with polyclonal rabbit anti-tau antibodies (Fig. 7C). The avidity of polyclonal rabbit anti-tau antibodies was higher for all three tau isoforms in contrast to nTau-Abs. Moreover, the signal intensity did not decrease by 50% in the range of 0–2.1 M NH_4SCN to evaluate avidity index for these His-tagged tau forms, tau 1–441 (the immunogen) and truncated tau 155–421. The avidity index of rabbit antibodies with truncated from tau 13–391 His-tag was determined as 1.36 M of NH_4SCN.

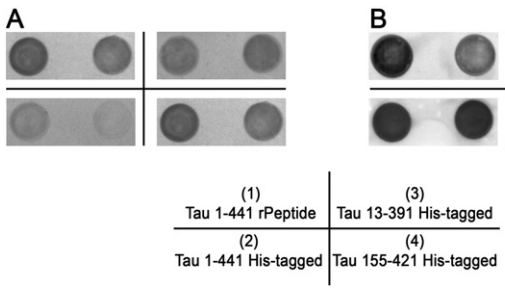


Fig. 4. Dot-blot immunoassay for evaluation the specific reactivity of isolated natural anti-tau Abs (A) and polyclonal rabbit anti-tau Abs (B) against tau with respect to used non-phosphorylated antigens: tau 1–441 rPeptide (1) and His-tagged tau forms: tau 1–441 (2), tau 13–391 (3), tau 155–421 (4) directly loaded onto PVDF membrane in the amount of 1 μg in left dot and 0.5 μg in right dot for each antigen. The formed immunocomplex was detected by Immun-Blot Opti-4CN Colorimetric kit.

4. Discussion

It is acknowledged that neurofibrillary tangle (NFT) formation is a dynamic process in which tau protein becomes modified and changes its conformational state. Amino acid site-specific phosphorylation and truncation at both N- and C-termini have been considered as the main post-translation modifications of tau protein in AD (Buee et al., 2000; Ding et al., 2006; Mondragon-Rodriguez et al., 2014; Novak, 1994). These post-translational modifications are able to significantly change the paperclip-like model of tau protein in a soluble form and promote the aggregation (Kovacech and Novak, 2010; Mandelkow et al., 2007; Wang et al., 2010). Truncation in C-terminal part of tau protein at cleavage points Asp421 (Fasulo et al., 2000; Gamblin et al., 2003) and Glu391 (Novak et al., 1993) was well defined and is even considered as a triggering factor in development of tau pathology (de Calignon et al., 2010; Garcia-Sierra et al., 2001; Mena et al., 1996; Rissman et al., 2004; Zilka et al., 2009). Moreover, the Asp421 cleavage always precedes Glu391 truncation in NFT formation (Basurto-Islas et al., 2008). To date, some facts are also known about the truncation in N-terminal part (Arai et al., 2005; Horowitz et al., 2004; Olesen, 1994). It was proven that truncation is a very early event in tangle formation, and that truncated tau phosphorylated at few specific sites even occurs before hyperphosphorylated form (Rissman et al., 2004). Some studies even postulated that phosphorylation of tau protein is a protective response

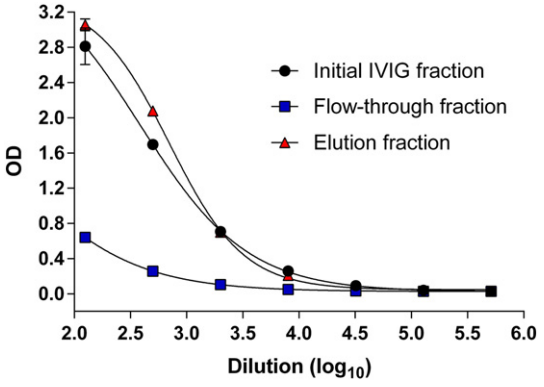


Fig. 5. Isolation efficiency of natural anti-tau antibodies from IVIG estimated by ELISA. Serial dilution of each purification fraction was probed against His-tagged tau 1–441 antigen. The detected signal is expressed as absorbance at wavelength 450 nm depending on the logarithm of samples dilution. His-tagged tau 1–441 form as antigen is coated onto a well in concentration 0.2 μg/50 μl in 0.1 M carbonate buffer pH 9.5. Initial Flebogamma IVIG fraction (50 mg/ml), flow-through fraction (IgG fraction passed through the column with covalently bound antigen tau 1–441 in His-tagged form with no retention, 7.9 mg/ml), elution fraction (IgG fraction retained in the column with covalently bound tau ligand, concentrated to final concentration 0.8 mg/ml due to transfer into PBS buffer by Amicon 30K filters) are illustrated.

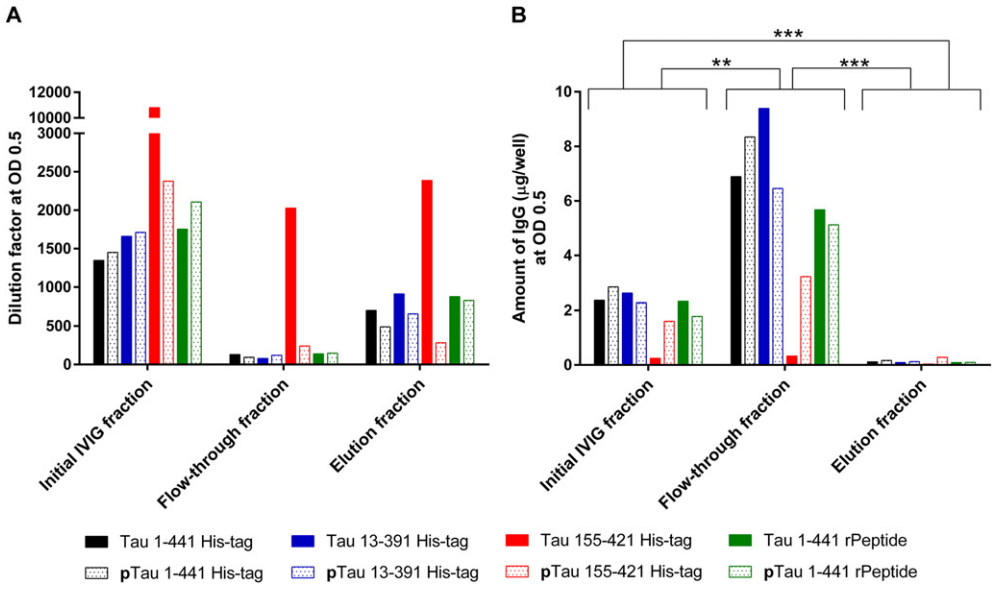


Fig. 6. Reactivity of natural Abs presented in individual IVIG purification fractions, initial Flebogamma IVIG fraction (50 mg/ml), flow-through fraction (7.9 mg/ml) and elution fraction (0.8 mg/ ml), against different forms of tau protein. The comparison is expressed as a dilution of each fraction (A) and the amount of IgG (B) required to obtain OD 0.5 at wavelength 450 nm. Bars represent mean value from duplicate wells. Statistical analysis was performed by Student t-test at significant p levels 0.01 (**), 0.001 (***).

to prevent the assembly of tau into filaments (Luna-Munoz et al., 2013; Schneider et al., 1999).

Tau protein occurs in numerous post-translationally modified forms in brain tissue. Especially site-specific phosphorylation and truncation are connected with the balance between physiological and pathological tau function. Thus, we included several tau forms varying in length and phosphorylation to characterize our isolated anti-tau antibodies naturally occurring in the commercial product of intravenous immunoglobulin Flebogamma. The IVIG is now in the spotlight because of ongoing clinical trials for AD therapeutic interventions. It has been proven that these products contain antibodies against neuronal tau protein (Smith et al., 2013) but their reactive character has been established only partly (Smith et al., 2014).

We have been able to isolate significant amount of these natural IgG molecules reacting with human recombinant full-length tau protein; namely 3.2 mg IgG molecules representing 0.8% of total amount of 400 mg IgG/8 ml Flebogamma IVIG product. To evaluate IgG subclass profile, we investigated the IgG subclass distribution in the initial IVIG product and the elution fraction. To our expectations, IgG1 constitutes the major subclass and IgG4 the last abundant subclass proportion in the two samples. We observed a slight decrease of IgG2 amount in the elution fraction in comparison to the initial IVIG product. Surprisingly, the result indicates skewing toward IgG3 in the elution fraction. The IgG3 subclass belongs to potent pro-inflammatory antibodies in early response to protein and viral antigens with a shorter half-life (Michaelsen et al., 2009; Vidarsson et al., 2014). It is also suggested that natural IgG Abs are IgG3 subclass-specific (Panda and Ding, 2015). Aforementioned findings may explain the low avidity profile of isolated antibodies reactive with tau protein. We used non-modified recombinant protein as a ligand for isolation of natural antibodies from IVIG to maximize the yield. We supposed that using post-translationally modified protein may decrease the isolation yield of Abs in detectable amounts. On the other hand, the amount of isolated antibodies against truncated and phosphorylated tau forms can be affected by using non-phosphorylated full-length tau as an antigen and these antibodies may not be sorted out during our isolation procedure. Thus, we would like to prepare more “native” like tau protein and repeat the isolation procedure as well.

The isolated naturally occurring anti-tau antibodies were subsequently characterized. Firstly, we would like to point out to results obtained with His-tagged tau 1–441 considering its phosphorylated state when comparing data from ELISA and Western blot. The

Table 1
Summary table of basic characterization of isolated natural anti-tau antibodies from Flebogamma IVIG product.

		Isolated natural anti-tau antibodies from Flebogamma IVIG against			
Antigen characteristics	Tau form	Tau 1–441 rPeptide	Tau 1–441 His-tagged	Tau 13–391 His-tagged	Tau 155–421 His-tagged
	His-Tag at NH_2-terminal position	−	+	+	+
Reactivity	Dot-blot immunoassay	++	+	++	+++
	Western blots *non-phosphorylated/phosphorylated*	++/+	++/±	+/+	++/+
	ELISA[a]:				
	Dilution (IgG amount in ng/well) *Non-phosphorylated/phosphorylated*	1:880 (93)/1:830 (97)	1:700 (114)/1:500 (169)	1:910 (88)/1:660 (121)	1:2400 (35)/1:290 (283)
Avidity index[b]	Dot-blot immunoassay	ND	0.74	0.46	1.67
	ELISA	ND	0.20	0.24	1.62

The qualitative level of isolated IgG molecules reactivity with different tau antigens loaded on PVDF membrane (dot-blot immunoassay) and transferred onto nitrocellulose membrane by semi-dry technique (Western blot) is illustrated as very weak (±), weak (+), intermediate (++) and strong (+++).
ND = not determined.
[a] ELISA results are expressed as a dilution of IgG molecules reacting with antigen (0.1 µg/well) to reach OD 0.5 at 450 nm (working concentration of isolated IgG molecules was 0.8 mg/ml).
[b] Avidity index is expressed as molarity of ammonium thiocyanate (M) that causes the decrease of initial sample signal (in the absence of thiocyanate) on the value of 50%.

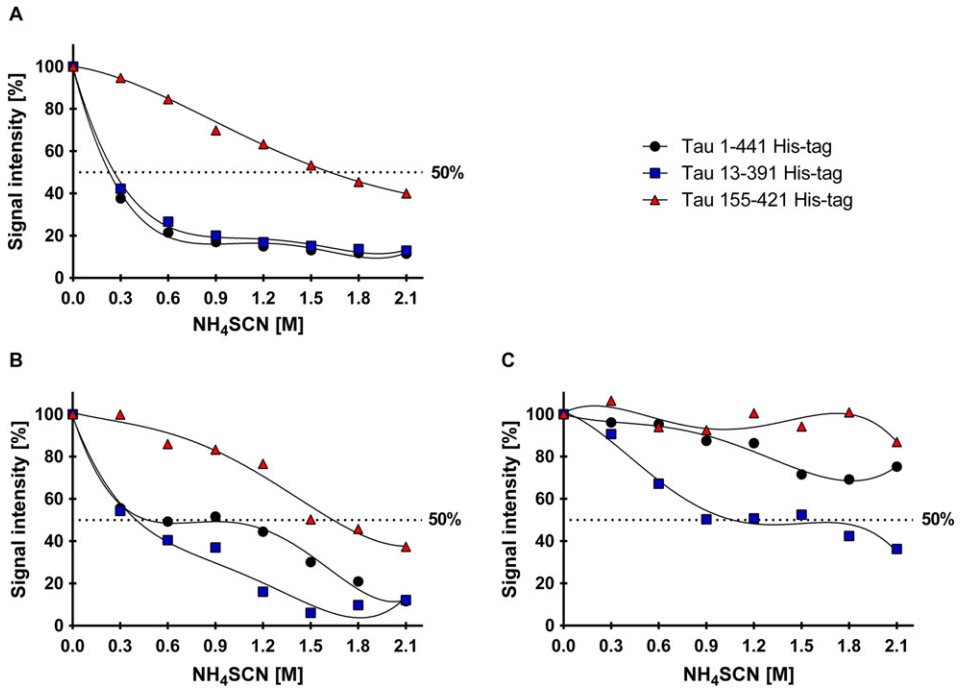


Fig. 7. Graphical representation of avidity index evaluation: expressed as the dependence of signal intensity (%; spot density in dot-blot immunoassay and absorbance at 450 nm in ELISA) to NH_4SCN molarity. Three tau isoforms were used: tau 1–441 His-tagged, tau 13–391 His-tagged and tau 155–421 His-tagged. Avidity of isolated natural anti-tau Abs from Flebogamma IVIG was measured by ELISA (A) and avidity dot-blot immunoassay (B). Additionally, avidity dot-blot immunoassay was carried out with polyclonal rabbit anti-tau Abs (C). Each point on the graph represents the mean value from wells in duplicates and spots in triplicates. Dotted line shows 50% decrease in signal intensity.

reactivity of isolated natural anti-tau Abs with phosphorylated tau 1–441 His-tag was significantly decreased in comparison with its non-phosphorylated form in Western blot analysis. In ELISA experiment, the reactivity difference between these two full-length tau forms was not as conspicuous as in blotting assays. We attribute this fact to a different level of protein structural quality changes during analysis which may substantially affect results revealing the immunoreactivity. In ELISA arrangement, the recombinant full-length tau containing His-tag has the conformation presumably closer to native state in comparison with altered conformation achieved by reducing SDS-PAGE electrophoresis prior to electrotransfer in Western blot.

One of the quotable findings in this study was that the nTau-Abs isolated from IVIG product containing IgG molecules pooled from several thousand healthy donors are not exclusively phospho-specific as they can recognize protein with and without this post-translational modification. This was verified by Western blot and ELISA. This corresponds with the study of Rosenmann's group in which no significant differences were observed in reactivity of circulating serum IgG with non-phosphorylated full-length tau and pathologically phosphorylated tau peptide (Rosenmann et al., 2006). We also took into consideration that the epitope preferences could be changed by acidic elution as was demonstrated in several publications (Djoumerska-Alexieva et al., 2010). On the other hand, we observed similar reactivity trends of natural antibodies in all fractions differing in buffer composition so we do not attribute great importance to acidic elution as the main factor of resulting reactivity of isolated nTau-Abs with various tau forms.

Interestingly, the occurrence of natural Abs reactive with phosphorylated and truncated tau forms, which are believed to be the main part of NFTs, was revealed by both ELISA and Western blot techniques. Some more remarkable results with a modified tau form, particularly with tau 155–421, were obtained. Especially, isolated anti-tau antibodies demonstrate the strongest signal on the spot with truncated form of His-tagged tau 155–421 in dot blot immunoassay. Moreover, in ELISA experiments we can observe that this truncated form has high antigenicity since all IVIG fractions (Initial, Flow-through, and Elution) showed strong reactivity against it. Therefore, this fragment may reveal or even bear some pathological features since the immune system shows such a strong response against it, which was also supported by highest avidity of isolated nTau-Abs to this fragment in contrast to other forms. This fragment contains all four repeat domains that are prone to aggregate as we have seen in Western blots. The latest studies have revealed that aggregated oligomeric tau forms are the toxic species of this protein. They can spread tau pathology and are responsible for cognitive impairment in AD mouse models which can be alleviated by passive immunotherapy specifically targeting tau oligomers (Castillo-Carranza et al., 2014a,b, 2015). In the future experiments would be, therefore, desirable to explore the reactivity of our polyclonal tau-reactive antibodies from IVIG products with thoroughly characterized oligomeric/aggregated tau forms. It was surprising that the reactivity of our nTau-Abs was partly abolished by phosphorylation since the phosphorylated, namely hyperphosphorylated, tau protein is contained in pathological NFTs in AD brains (Iqbal et al., 2005).

5. Conclusions

To conclude our study, we proved the existence of specific natural anti-tau Abs in plasma IVIG from healthy donors that could refer to immune involvement as a regulatory mechanism of modified and toxic tau fragment occurrence. More experiments are needed to evaluate our hypothesis. For instance, it would be interesting to compare the reactivity profile of antibodies isolated from other IVIG products or even from pooled AD samples with the reactivity of these isolated antibodies from Flebogamma IVIG product. Nowadays, it is also discussed if crude IVIG products or only specific Abs isolated from IVIG against particular antigenic epitopes should be used for therapeutic approaches. Therefore, epitope mapping of these target-reactive Abs could also be helpful.

Acknowledgments

This work was supported by the research project GACR P304/12/G069, by the "National Institute of Mental Health (NIMH-CZ)", grant

number CZ.1.05/2.1.00/03.0078 and the European Regional Development Fund, by EU project NADINE (No. 246513), and by the Ministry of Education, Youth and Sports of the Czech Republic (project CZ.1.07/2.3.00/30.0021 "Enhancement of R&D Pools of Excellence at the University of Pardubice").

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2015.10.017.

References

Arai, T., Guo, J.-P., McGeer, P.L., 2005. Proteolysis of non-phosphorylated and phosphorylated tau by thrombin. J. Biol. Chem. 280, 5145–5153. http://dx.doi.org/10.1074/jbc.M409234200.

Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T., Hyman, B.T., 1992a. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 42, 631–639.

Arriagada, P.V., Marzloff, K., Hyman, B.T., 1992b. Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease. Neurology 42, 1681–1688.

Avrameas, S., Ternynck, T., 1993. The natural autoantibodies system: between hypotheses and facts. Mol. Immunol. 30, 1133–1142. http://dx.doi.org/10.1016/0161-5890(93)90160-D.

Bahmanyar, S., Moreau-Dubois, M.-C., Brown, P., Cathala, F., Gajdusek, D.C., 1983. Serum antibodies to neurofilament antigens in patients with neurological and other diseases and in healthy controls. J. Neuroimmunol. 5, 191–196. http://dx.doi.org/10.1016/0165-5728(83)90009-7.

Balakrishnan, K., Andrei-Selmer, L.-C., Selmer, T., Bacher, M., Dodel, R., 2010. Comparison of intravenous immunoglobulins for naturally occurring autoantibodies against amyloid-β. J. Alzheimers Dis. 20, 135–143. http://dx.doi.org/10.3233/JAD-2010-1353.

Ballow, M., 2009. Clinical experience with Flebogamma 5% DIF: a new generation of intravenous immunoglobulins in patients with primary immunodeficiency disease. Clin. Exp. Immunol. 157 (Suppl. 1), 22–25. http://dx.doi.org/10.1111/j.1365-2249.2009.03951.x.

Bartos, A., Fialova, L., Svarcova, J., Ripova, D., 2012. Patients with Alzheimer disease have elevated intrathecal synthesis of antibodies against tau protein and heavy neurofilament. J. Neuroimmunol. 252, 100–105. http://dx.doi.org/10.1016/j.jneuroim.2012.08.001.

Basurto-Islas, G., Luna-Munoz, J., Guillozet-Bongaarts, A.L., Binder, L.I., Mena, R., Garcia-Sierra, F., 2008. Accumulation of aspartic acid421- and glutamic acid391-cleaved tau in neurofibrillary tangles correlates with progression in Alzheimer disease. J. Neuropathol. Exp. Neurol. 67, 470–483. http://dx.doi.org/10.1097/NEN.0b013e31817275c7.

Braak, E., Griffing, K., Arai, K., Bohl, J., Bratzke, H., Braak, H., 1999. Neuropathology of Alzheimer's disease: what is new since A. Alzheimer? Eur. Arch. Psychiatry Clin. Neurosci. 249 (Suppl. 3), 14–22.

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., Hof, P.R., 2000. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res. Brain Res. Rev. 33, 95–130.

Carrell, R.W., Lomas, D.A., 1997. Conformational disease. Lancet 350, 134–138. http://dx.doi.org/10.1016/S0140-6736(97)02073-4.

Castillo-Carranza, D.L., Gerson, J.E., Sengupta, U., Guerrero-Munoz, M.J., Lasagna-Reeves, C.A., Kayed, R., 2014a. Specific targeting of tau oligomers in Htau mice prevents cognitive impairment and tau toxicity following injection with brain-derived tau oligomeric seeds. J. Alzheimers Dis. JAD 40 (Suppl. 1), S97–S111. http://dx.doi.org/10.3233/JAD-132477.

Castillo-Carranza, D.L., Sengupta, U., Guerrero-Munoz, M.J., Lasagna-Reeves, C.A., Gerson, J.E., Singh, G., Estes, D.M., Barrett, A.D.T., Dineley, K.T., Jackson, G.R., Kayed, R., 2014b. Passive immunization with Tau oligomer monoclonal antibody reverses tauopathy phenotypes without affecting hyperphosphorylated neurofibrillary tangles. J. Neurosci. Off. J. Soc. Neurosci. 34, 4260–4272. http://dx.doi.org/10.1523/JNEUROSCI.3192-13.2014.

Castillo-Carranza, D.L., Guerrero-Munoz, M.J., Sengupta, U., Hernandez, C., Barrett, A.D.T., Dineley, K., Kayed, R., 2015. Tau immunotherapy modulates both pathological tau and upstream amyloid pathology in an Alzheimer's disease mouse model. J. Neurosci. Off. J. Soc. Neurosci. 35, 4857–4868. http://dx.doi.org/10.1523/JNEUROSCI.4989-14.2015.

de Calignon, A., Fox, L.M., Pitstick, R., Carlson, G.A., Bacskai, B.J., Spires-Jones, T.L., Hyman, B.T., 2010. Caspase activation precedes and leads to tangles. Nature 464, 1201–1204. http://dx.doi.org/10.1038/nature08890.

Ding, H., Matthews, T.A., Johnson, G.V.W., 2006. Site-specific phosphorylation and caspase cleavage differentially impact tau-microtubule interactions and tau aggregation. J. Biol. Chem. 281, 19107–19114. http://dx.doi.org/10.1074/jbc.M511697200.

Djoumerska-Alexieva, I.K., Dimitrov, J.D., Voynova, E.N., Lacroix-Desmazes, S., Kaveri, S.V., Vassilev, T.L., 2010. Exposure of IgG to an acidic environment results in molecular modifications and in enhanced protective activity in sepsis. FEBS J. 277, 3039–3050. http://dx.doi.org/10.1111/j.1742-4658.2010.07714.x.

Dodel, R., Hampel, H., Depboylu, C., Lin, S., Gao, F., Schock, S., Jackel, S., Wei, X., Buerger, K., Hoft, C., Hemmer, B., Moller, H.-J., Farlow, M., Oertel, W.H., Sommer, N., Du, Y., 2002. Human antibodies against amyloid β peptide: a potential treatment for Alzheimer's disease. Ann. Neurol. 52, 253–256. http://dx.doi.org/10.1002/ana.10253.

Du, Y., Wei, X., Dodel, R., Sommer, N., Hampel, H., Gao, F., Ma, Z., Zhao, L., Oertel, W.H., Farlow, M., 2003. Human anti-β-amyloid antibodies block β-amyloid fibril formation and prevent β-amyloid-induced neurotoxicity. Brain 126, 1935–1939. http://dx.doi.org/10.1093/brain/awg191.

Fasler, S., Skvaril, F., Lutz, H.U., 1988. Electrophoretic properties of human IgG and its subclasses on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblots. Anal. Biochem. 174, 593–600.

Fasulo, L., Ugolini, G., Visintin, M., Bradbury, A., Brancolini, C., Verzillo, V., Novak, M., Cattaneo, A., 2000. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. J. Neurochem. 75, 624–633.

Fuchs, S., Feferman, T., Meidler, R., Margalit, R., Sicsic, C., Wang, N., Zhu, K.-Y., Brenner, T., Laub, O., Souroujon, M.C., 2008. A disease-specific fraction isolated from IVIG is essential for the immunosuppressive effect of IVIG in experimental autoimmune myasthenia gravis. J. Neuroimmunol. 194, 89–96. http://dx.doi.org/10.1016/j.jneuroim.2007.11.020.

Gamblin, T.C., Chen, F., Zambrano, A., Abraha, A., Lagalwar, S., Guillozet, A.L., Lu, M., Fu, Y., Garcia-Sierra, F., LaPointe, N., Miller, R., Berry, R.W., Binder, L.I., Cryns, V.L., 2003. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 100, 10032–10037. http://dx.doi.org/10.1073/pnas.1630428100.

Garcia-Sierra, F., Wischik, C.M., Harrington, C.R., Luna-Munoz, J., Mena, R., 2001. Accumulation of C-terminally truncated tau protein associated with vulnerability of the perforant pathway in early stages of neurofibrillary pathology in Alzheimer's disease. J. Chem. Neuroanat. 22, 65–77.

Garcia-Sierra, F., Ghoshal, N., Quinn, B., Berry, R.W., Binder, L.I., 2003. Conformational changes and truncation of tau protein during tangle evolution in Alzheimer's disease. J. Alzheimers Dis. JAD 5, 65–77.

Garcia-Sierra, F., Mondragón-Rodriguez, S., Basurto-Islas, G., 2008. Truncation of tau protein and its pathological significance in Alzheimer's disease. J. Alzheimers Dis. JAD 14, 401–409.

Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J.H., Petersen, R.C., Parisi, J.E., Hyman, B.T., 1997. Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann. Neurol. 41, 17–24. http://dx.doi.org/10.1002/ana.410410106.

Grifols Biologicals Inc., 2015. ClinicalTrials.gov: a study to evaluate albumin and immunoglobulin in Alzheimer's disease (AMBAR) In https://clinicaltrials.gov/show/NCT01561053.

Guillozet-Bongaarts, A.L., Garcia-Sierra, F., Reynolds, M.R., Horowitz, P.M., Fu, Y., Wang, T., Cahill, M.E., Bigio, E.H., Berry, R.W., Binder, L.I., 2005. Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. Neurobiol. Aging 26, 1015–1022. http://dx.doi.org/10.1016/j.neurobiolaging.2004.09.019.

Hanger, D.P., Wray, S., 2010. Tau cleavage and tau aggregation in neurodegenerative disease. Biochem. Soc. Trans. 38, 1016–1020. http://dx.doi.org/10.1042/BST0381016.

Hermanson, G.T., 2013. Bioconjugate Techniques. Academic Press.

Horowitz, P.M., Patterson, K.R., Guillozet-Bongaarts, A.L., Reynolds, M.R., Carroll, C.A., Weintraub, S.T., Bennett, D.A., Cryns, V.L., Berry, R.W., Binder, L.I., 2004. Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease. J. Neurosci. Off. J. Soc. Neurosci. 24, 7895–7902. http://dx.doi.org/10.1523/JNEUROSCI.1988-04.2004.

Iqbal, K., del C. Alonso, A., Chen, S., Chohan, M.O., El-Akkad, E., Gong, C.-X., Khatoon, S., Li, B., Liu, F., Rahman, A., Tanimukai, H., Grundke-Iqbal, I., 2005. Tau pathology in Alzheimer disease and other tauopathies. Biochim. Biophys. Acta BBA Mol. Basis Dis. Biol. Pathobiol. Tau 1739, 198–210. http://dx.doi.org/10.1016/j.bbadis.2004.09.008.

Jianping, L., Zhibing, Y., Wei, Q., Zhikai, C., Jie, X., Jinbiao, L., 2006. Low avidity and level of serum anti-Abeta antibodies in Alzheimer disease. Alzheimer Dis. Assoc. Disord. 20, 127–132.

Jorquera, J.I., 2009. Flebogamma® 5% DIF development: rationale for a new option in intravenous immunoglobulin therapy. Clin. Exp. Immunol. 157, 17–21. http://dx.doi.org/10.1111/j.1365-2249.2009.03953.x.

Kajii, M., Kobayashi, F., Kashihara, J., Yuuki, T., Kubo, Y., Nakae, T., Kamizono, A., Kuzumoto, Y., Kusunoki, S., 2014. Intravenous immunoglobulin preparation attenuates neurological signs in rat experimental autoimmune neuritis with the suppression of macrophage inflammatory protein-1α expression. J. Neuroimmunol. 266, 43–48. http://dx.doi.org/10.1016/j.jneuroim.2013.10.011.

Kayed, R., Jackson, G.R., Estes, D.M., Barrett, A.D.T., 2011. Alzheimers disease: review of emerging treatment role for intravenous immunoglobulins. J. Cent. Nerv. Syst. Dis. 3, 67–73. http://dx.doi.org/10.4137/JCNSD.S5018.

Klaver, A.C., Finke, J.M., Digambaranath, J., Balasubramaniam, M., Loeffler, D.A., 2010a. Antibody concentrations to Aβ1–42 monomer and soluble oligomers in untreated and antibody–antigen-dissociated intravenous immunoglobulin preparations. Int. Immunopharmacol. 10, 115–119. http://dx.doi.org/10.1016/j.intimp.2009.10.005.

Klaver, A.C., Patrias, L.M., Coffey, M.P., Finke, J.M., Loeffler, D.A., 2010b. Measurement of anti-Aβ1–42 antibodies in intravenous immunoglobulin with indirect ELISA: the problem of nonspecific binding. J. Neurosci. Methods 187, 263–269. http://dx.doi.org/10.1016/j.jneumeth.2010.01.018.

Klaver, A.C., Coffey, M.P., Smith, L.M., Loeffler, D.A., 2013. Comparison of ELISA measurements of anti-Aβ concentrations and percentages of specific binding to Aβ between unfractionated intravenous immunoglobulin products and their purified anti-Aβ antibodies. Immunol. Lett. 154, 7–11. http://dx.doi.org/10.1016/j.imlet.2013.07.008.

Kolarova, M., Garcia-Sierra, F., Bartos, A., Ricny, J., Ripova, D., 2012. Structure and pathology of tau protein in Alzheimer disease. Int. J. Alzheimers Dis. 2012, 731526. http://dx.doi.org/10.1155/2012/731526.

Kovacech, B., Novak, M., 2010. Tau truncation is a productive posttranslational modification of neurofibrillary degeneration in Alzheimer's disease. Curr. Alzheimer Res. 7, 708–716.

Kristofikova, Z., Ricny, J., Kolarova, M., Vyhnalek, M., Hort, J., Laczo, J., Sirova, J., Ripova, D., 2014. Interactions between amyloid-β and tau in cerebrospinal fluid of people with mild cognitive impairment and Alzheimer's disease. J. Alzheimers Dis. JAD 42 (Suppl. 3), S91–S98. http://dx.doi.org/10.3233/JAD-132393.

Levin, E.C., Acharya, N.K., Han, M., Zavareh, S.B., Sedeyn, J.C., Venkataraman, V., Nagele, R.G., 2010. Brain-reactive autoantibodies are nearly ubiquitous in human sera and may be linked to pathology in the context of blood–brain barrier breakdown. Brain Res. 1345, 221–232. http://dx.doi.org/10.1016/j.brainres.2010.05.038.

Luna-Munoz, J., R., C, M., C, Flores-Rodriguez, P., Avila, J., R., S, la Cruz, F.D., Mena, R., A., M, Floran-Garduno, B., 2013. Phosphorylation of tau protein associated as a protective mechanism in the presence of toxic, C-terminally truncated tau in Alzheimer's Disease. In: Zerr, I. (Ed.), Understanding Alzheimer's Disease. InTech.

Mandelkow, E., von Bergen, M., Biernat, J., Mandelkow, E.-M., 2007. Structural principles of tau and the paired helical filaments of Alzheimer's disease. Brain Pathol. 17, 83–90. http://dx.doi.org/10.1111/j.1750-3639.2007.00053.x.

Mena, R., Edwards, P.C., Harrington, C.R., Mukaetova-Ladinska, E.B., Wischik, C.M., 1996. Staging the pathological assembly of truncated tau protein into paired helical filaments in Alzheimer's disease. Acta Neuropathol. (Berl.) 91, 633–641. http://dx.doi.org/10.1007/s004010050477.

Michaelsen, T.E., Sandlie, I., Bratlie, D.B., Sandin, R.H., Ihle, O., 2009. Structural difference in the complement activation site of human IgG1 and IgG3. Scand. J. Immunol. 70, 553–564. http://dx.doi.org/10.1111/j.1365-3083.2009.02338.x.

Miescher, S.M., Schaub, A., Ghielmetti, M., Baumann, M., Vogel, M., Bolli, R., Stadler, B., 2005. Comparative analysis of antigen specificities in the monomeric and dimeric fractions of intravenous immunoglobulin. Ann. N. Y. Acad. Sci. 1051, 582–590. http://dx.doi.org/10.1196/annals.1361.102.

Mondragon-Rodriguez, S., Perry, G., Luna-Munoz, J., Acevedo-Aquino, M.C., Williams, S., 2014. Phosphorylation of tau protein at sites Ser396–404 is one of the earliest events in Alzheimer's disease and Down syndrome. Neuropathol. Appl. Neurobiol. 40, 121–135. http://dx.doi.org/10.1111/nan.12084.

Novak, M., 1994. Truncated tau protein as a new marker for Alzheimer's disease. Acta Virol. 38, 173–189.

Novak, M., Kabat, J., Wischik, C.M., 1993. Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. EMBO J. 12, 365–370.

Olesen, O.F., 1994. Proteolytic degradation of microtubule associated protein tau by thrombin. Biochem. Biophys. Res. Commun. 201, 716–721. http://dx.doi.org/10.1006/bbrc.1994.1759.

Panda, S., Ding, J.L., 2015. Natural antibodies bridge innate and adaptive immunity. J. Immunol. 194, 13–20. http://dx.doi.org/10.4049/jimmunol.1400844.

Pul, R., Dodel, R., Stangel, M., 2011. Antibody-based therapy in Alzheimer's disease. Expert. Opin. Biol. Ther. 11, 343–357. http://dx.doi.org/10.1517/14712598.2011.552884.

Pullen, G.R., Fitzgerald, M.G., Hosking, C.S., 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods 86, 83–87. http://dx.doi.org/10.1016/0022-1759(86)90268-1.

Rissman, R.A., Poon, W.W., Blurton-Jones, M., Oddo, S., Torp, R., Vitek, M.P., LaFerla, F.M., Rohn, T.T., Cotman, C.W., 2004. Caspase-cleavage of tau is an early event in Alzheimer disease tangle pathology. J. Clin. Invest. 114, 121–130. http://dx.doi.org/10.1172/JCI200420640.

Rosenmann, H., Meiner, Z., Geylis, V., Abramsky, O., Steinitz, M., 2006. Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. Neurosci. Lett. 410, 90–93. http://dx.doi.org/10.1016/j.neulet.2006.01.072.

Schneider, A., Biernat, J., von Bergen, M., Mandelkow, E., Mandelkow, E.M., 1999. Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. Biochemistry (Mosc) 38, 3549–3558. http://dx.doi.org/10.1021/bi981874p.

Sedykh, M.A., Buneva, V.N., Nevinsky, G.A., 2013. Polyreactivity of natural antibodies: exchange by HL-fragments. Biochem. Mosc. 78, 1305–1320. http://dx.doi.org/10.1134/S0006297913120018.

Seite, J.-F., Shoenfeld, Y., Youinou, P., Hillion, S., 2008. What is the contents of the magic draft IVIg? Autoimmun. Rev. Naturally Occurring Antibodies Health Dis. 7, 435–439. http://dx.doi.org/10.1016/j.autrev.2008.04.012.

Smith, L.M., Coffey, M.P., Klaver, A.C., Loeffler, D.A., 2013. Intravenous immunoglobulin products contain specific antibodies to recombinant human tau protein. Int. Immunopharmacol. 16, 424–428. http://dx.doi.org/10.1016/j.intimp.2013.04.034.

Smith, L.M., Coffey, M.P., Loeffler, D.A., 2014. Specific binding of intravenous immunoglobulin products to tau peptide fragments. Int. Immunopharmacol. 21, 279–282. http://dx.doi.org/10.1016/j.intimp.2014.05.009.

Soto, C., 1999. Alzheimer's and prion disease as disorders of protein conformation: implications for the design of novel therapeutic approaches. J. Mol. Med. 77, 412–418. http://dx.doi.org/10.1007/s001090050371.

Stangel, D.M., Pul, R., 2006. Basic principles of intravenous immunoglobulin (IVIg) treatment. J. Neurol. 253, v18–v24. http://dx.doi.org/10.1007/s00415-006-5003-1.

Svobodova, et al., 2013. Dot-ELISA affinity test: an easy, low-cost method to estimate binding activity of monoclonal antibodies. J. Anal. Bioanal. Tech. 04. http://dx.doi.org/10.4172/2155-9872.1000168.

Szabo, P., Relkin, N., Weksler, M.E., 2008. Natural human antibodies to amyloid beta peptide. Autoimmun. Rev. Naturally Occurring Antibodies Health Dis. 7, 415–420. http://dx.doi.org/10.1016/j.autrev.2008.03.007.

Szabo, P., Mujalli, D.M., Rotondi, M.L., Sharma, R., Weber, A., Schwarz, H.-P., Weksler, M.E., Relkin, N., 2010. Measurement of anti-beta amyloid antibodies in human blood. J. Neuroimmunol. 227, 167–174. http://dx.doi.org/10.1016/j.jneuroim.2010.06.010.

Terryberry, J.W., Thor, G., Peter, J.B., 1998. Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis. Neurobiol. Aging 19, 205–216. http://dx.doi.org/10.1016/S0197-4580(98)00049-9.

Vassilev, T.L., Bineva, I.L., Dietrich, G., Kaveri, S.V., Kazatchkine, M.D., 1995. Variable region-connected, dimeric fraction of intravenous immunoglobulin enriched in natural autoantibodies. J. Autoimmun. 8, 405–413. http://dx.doi.org/10.1006/jaut.1995.0032.

Vidarsson, G., Dekkers, G., Rispens, T., 2014. IgG subclasses and allotypes: from structure to effector functions. Front. Immunol. 5. http://dx.doi.org/10.3389/fimmu.2014.00520.

Wang, Y., Garg, S., Mandelkow, E.-M., Mandelkow, E., 2010. Proteolytic processing of tau. Biochem. Soc. Trans. 38, 955–961. http://dx.doi.org/10.1042/BST0380955.

Watts, H., Kennedy, P.G.E., Thomas, M., 1981. The significance of anti-neuronal antibodies in Alzheimer's disease. J. Neuroimmunol. 1, 107–116. http://dx.doi.org/10.1016/0165-5728(81)90013-8.

Wymann, S., Ghielmetti, M., Schaub, A., Baumann, M.J., Stadler, B.M., Bolli, R., Miescher, S.M., 2008. Monomerization of dimeric IgG of intravenous immunoglobulin (IVIg) increases the antibody reactivity against intracellular antigens. Mol. Immunol. 45, 2621–2628. http://dx.doi.org/10.1016/j.molimm.2007.12.020.

Zilka, N., Stozicka, Z., Kovac, A., Pilipcinec, E., Bugos, O., Novak, M., 2009. Human misfolded truncated tau protein promotes activation of microglia and leukocyte infiltration in the transgenic rat model of tauopathy. J. Neuroimmunol. 209, 16–25. http://dx.doi.org/10.1016/j.jneuroim.2009.01.013.

Publication III:

Characterization of isolated tau-reactive antibodies from the IVIG product, plasma of patients with Alzheimer's disease and cognitively normal individuals.

Krestova, M., Hromadkova, L., Bilkova, Z., Bartos, A., Ricny, J., Journal of Neuroimmunology, 2017, 313, 16-24.

Stated contributions of the author: Experimental part (SDS-PAGE electrophoresis, tau phosphorylation, blotting techniques, avidity dot-blot immunoassay, and antibodies´ biotinylation). Revision of the manuscript.

Journal of Neuroimmunology 313 (2017) 16–24

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Characterization of isolated tau-reactive antibodies from the IVIG product, plasma of patients with Alzheimer's disease and cognitively normal individuals

Michala Krestova[a,b], Lenka Hromadkova[a,c,d,*], Zuzana Bilkova[d], Ales Bartos[a,b], Jan Ricny[a]

[a] National Institute of Mental Health, Klecany, Czech Republic
[b] Third Faculty of Medicine, Charles University, Prague, Czech Republic
[c] Faculty of Science, Charles University, Prague, Czech Republic
[d] Department of Biological and Biochemical Sciences, University of Pardubice, Pardubice, Czech Republic

ARTICLE INFO

Keywords:
Alzheimer's disease
Brain homogenates
Plasma antibodies
Reactivity profile
Tau protein

ABSTRACT

The presence of natural tau-reactive antibodies was reported in human blood. In this study, we isolated and characterized natural tau-reactive antibodies occurring in IVIG product Flebogamma, plasma of patients with Alzheimer's disease (AD) and older cognitively normal persons (controls). Using blotting immunoassays and ELISA, we showed reactivity of antibodies obtained from IVIG and controls against a recombinant fragment of tau (155–421 aa) and aggregates present in brains of AD patients. In contrast, antibodies isolated from plasma of AD patients reacted mainly with the recombinant full-length tau form and tau monomeric forms in brain tissue.

1. Introduction

Nowadays, the therapeutic potential of antibodies in neurodegenerative diseases is being studied quite intensively (Schroeder et al., 2016). Several research groups have focused on the treatment of Alzheimer's disease (AD) using specific monoclonal antibodies, polyclonal intravenous immunoglobulins (IVIG) and vaccines (Castillo-Carranza et al., 2015; Davtyan et al., 2016; Dodel et al., 2004; Gu et al., 2013; Kayed, 2010; Knight and Gandy, 2014; Kontsekova et al., 2014; Sigurdsson, 2009; Steinitz, 2009). The IVIG products attracted a lot of attention as a possible treatment of neurological disorders (Fuchs et al., 2008) and even AD (Dodel et al., 2010; Kile et al., 2015; Neff et al., 2008; Relkin, 2014). However, the clinical trials for treatment of AD failed for two of the IVIG products, Octagam and Gammagard (Baxter U.S., 2013a, 2013b; Dodel et al., 2013). Another two clinical trials with Flebogamma® from Grifols (AMBAR, phase III, NCT01561053) and Sutter Health's IVIG NewGam™ (phase II, NCT01300728) are still under investigations (Grifols Biologicals Inc., 2015; Kile et al., 2015). However, the NewGam™ IVIG trial reported that a short course of IVIG administered in the MCI stage of AD reduces brain atrophy, prevents cognitive decline in late stage of MCI and delays conversion to AD dementia for at least 1 year (Kile et al., 2015). In the light of these findings, the characterization of reactivity of specific antibodies against either amyloid beta or tau protein present in these products (Balakrishnan et al., 2010; Dodel et al., 2004; Klaver et al., 2013; Smith et al., 2014, 2013) could clarify their contribution to the effect of these treatments. Notably, when we consider the use of AD-specific IVIG preparations enriched by antibodies specific for tau, amyloid-beta, complement, cytokines and other factors as was discussed by (Loeffler, 2014).

In the previous work, we showed specificity of plasma antibodies obtained from Flebogamma IVIG product against fragment 155–421 aa of tau protein (Hromadkova et al., 2015). The fragment 155–421 aa is cleaved at the Asp421 which is an early event in the pathological assembly of truncated tau in the neurofibrillary tangles (NFTs) evolution (Basurto-Islas et al., 2008). Moreover, tau comprises thrombin cleavage site at Arg155 and the products of thrombin proteolysis are potentially pathogenic (Chesser et al., 2013; Hanger and Wray, 2010) and both thrombin and its precursor prothrombin, are expressed by neurons and glia, and accumulate in NFTs in AD (Arai et al., 2006). Thus, our findings point towards the involvement of immune system in controlling the occurrence of pathological proteins under physiological conditions. However, an investigation of the characteristics and reactivity of naturally occurring antibodies in plasma against native physiological and pathological forms of tau protein is needed. In this study, we used homogenates of brain tissue from AD patients and control subject to reflect the reactivity of naturally occurring antibodies against native forms of tau protein. Moreover, in spite of the effort, little is known

* Corresponding author at: National Institute of Mental Health, Klecany, Czech Republic.
E-mail addresses: michala.kolarova@nudz.cz (M. Krestova), lenka.hromadkova@nudz.cz (L. Hromadkova), Zuzana.Bilkova@upce.cz (Z. Bilkova), ales.bartos@nudz.cz (A. Bartos), jan.ricny@nudz.cz (J. Ricny).

http://dx.doi.org/10.1016/j.jneuroim.2017.09.011
Received 13 April 2017; Received in revised form 20 June 2017; Accepted 27 September 2017
0165-5728/ © 2017 Elsevier B.V. All rights reserved.

about the naturally occurring tau-reactive antibody profile in serum of patients with diagnosed AD and old, but cognitively normal individuals. Therefore, we looked into the reactivity profiles of antibodies found in the pool of plasma samples from patients with diagnosed AD and age-matched cognitively normal individuals with non-inflammatory neurological diseases. We isolated three fractions of natural tau-reactive antibodies from IVIG (nTau-IVIG), plasma of AD patients (nTau-AD) and cognitively normal control subjects (nTau-Ctrl). Their reactivity was investigated by ELISA and blotting techniques against recombinant non-phosphorylated/phosphorylated full-length (1–441 aa) form and fragment (155–421 aa) of tau protein and native tau proteins present in the homogenates of brain tissue.

2. Materials and methods

2.1. Participants

The research was approved by the Ethics Committee of the University Hospital Kralovske Vinohrady and conducted according to the Declaration of Helsinki and the Laws 129/2003 and 130/2003 of the Czech Republic. Informed consent was obtained from all individual participants included in this study.

Plasma samples were obtained from 11 patients from the Department of Neurology or Memory Clinic of the Charles University, Czech Republic. Their cognitive functions were evaluated using an updated Czech version of Addenbrooke's Cognitive Examination Revised (ACE-CZ) (Bartoš et al., 2011; Mioshi et al., 2006). We were then able to derive MMSE scores (range 0–30) from the ACE-CZ (score range 0–100). Four aged men and three elderly women were recruited as in-patients from the Department of Neurology and classified as cognitively normal. They had normal ACE-CZ (cut-off ≥ 79) and MMSE scores (cut-off ≥ 28). They mostly presented with non-inflammatory conditions such as polyneuropathy and peripheral Bell's facial palsy and the rest presented with a variety of diseases (e.g. a headache, trigeminal neuralgia and transient unconsciousness). Four AD patients (two men and two women) were diagnosed according to the NIA-AA criteria (McKhann et al., 2011).

Plasma samples were collected, centrifuged, and aliquoted in 1 ml polypropylene tubes and stored (on average within 1.5 h of sampling) at − 80 °C until analysis. The specimens were thawed just before experiments. All participants signed an informed consent.

Human brain tissues of one control individual (79 years old man whose cause of death was myocardial infarct) and two AD patients (two men at the age of 82 and 83 whose cause of death was cardiac insufficiency) were obtained by autopsy. Brains were evaluated using silver staining technique, dissected and stored according to the study of (Kriştofikova et al., 1995). The control subject was described as a nondemented and nonpsychotic patient (no marked histological changes indicative of AD pathology). The AD patients had clinically diagnosed dementia with a number of senile plaques and tangles in given areas of the cortex and in the hippocampus higher than would be expected for age. The criteria for the postmortem assessment of dementia and control subjects were consistent with those standardized by Dr. S. S. Mirra et al. from VA Medical Center, Department of Pathology and Laboratory Medicine (Decatur, GA) (Mirra et al., 1991).

2.2. Preparation of tau protein forms

2.2.1. Recombinant tau forms

The reactivity of isolated anti-tau antibodies was examined by using four recombinant His-tagged tau forms. The preparation of His-tagged full-length human tau 1–441 amino acids (tau 1–441 aa) and His-tagged human truncated tau form (tau 155–421 aa) was performed as previously described (Hromadkova et al., 2015). Both tau forms (52 µg each) were also phosphorylated by kinase mixture (500 U of GSK-3 from New England Biolabs (Ipswich, MA, USA), 125 U of ERK2 and 750 U of PKA from Biaffin GmbH & Co KG (Kassel, Germany)) in 200 µl of 40 mM HEPES buffer pH 7.2 with 5 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, and 1 mM ATP for 20 h at 30 °C. The phosphorylation was stopped by heat-denaturation of kinases (95 °C, 10 min). The phosphorylation state of tau proteins was confirmed by a mobility shift in SDS-polyacrylamide gel and by Western blots with specific anti-phospho-tau antibodies as described previously (Hromadkova et al., 2015). For all subsequent experiments, tau protein forms were transferred into PBS buffer by using Amicon® Ultra 0.5 ml filters (10 K, Merck Millipore, Billerica, MA, USA).

2.2.2. Tau protein forms in brain tissue homogenates

The reactivity of isolated anti-tau antibodies was also investigated using human brain homogenates where tau protein occurs in various native forms. The detailed characterization of the three used brain samples is summarized in the section 'Participants'. Sections of left hemisphere hippocampi from two AD patients and one control were homogenized in 1 × PBS buffer containing 2 × inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), 1 mM EDTA and 0.02% NaN_3 or with additional 2% SDS in 1:3 w/v dilution followed by 10,000 × g spin. Supernatants were collected and the total protein content of all homogenized samples was determined by BCA test according to manufacturer's instructions (Thermo Scientific, Waltman, MA, USA).

2.3. Isolation of natural tau-reactive antibodies

The antibodies were purified against tau protein by low-pressure affinity chromatography as previously described (Hromadkova et al., 2015). New column was prepared as follows: 4 mg of His-tagged tau 1–441 aa protein was reacted with 2 ml of pre-packed resin (Labiomer 300, epoxy-activated, 50 µm bead size in Econo-Pac column of 14 cm length and 1.5 cm diameter), residual reactive epoxide groups were blocked with 0.2 M ethanolamine overnight at 4 °C and then the column was equilibrated with PBS buffer (Hermanson, 2013). IVIG (3 ml, Flebogamma® 5% DIF (5 g/100 ml), Grifols Biologicals Inc., Los Angeles, CA, USA), plasma pool of samples from seven cognitively normal control subjects (3 ml; 108.5 mg/ml) and four patients with AD (3.5 ml; 107.3 mg/ml) were diluted to 10 ml in PBS and loaded onto the column. General characteristics of donors of plasma samples are listed in Table 1. The amount of protein in the initial fractions, flow-through (F-T) fractions and concentrated eluted fractions (before the addition of glycerol) was determined by BCA assay according to manufacturer's instructions (Thermo Scientific, Waltman, MA, USA).

Table 1
General characteristics of donors of plasma samples.

Groups	n	Sex (M/F)	Age (years)	MMSE (0 − 30)	ACE (0 − 100)	$A\beta_{42}$ (pg/ml)	Total Tau (pg/ml)	Phospho-Tau_{181} (pg/ml)
Controls	7	4/3	70 ± 8	28.5 ± 1	88 ± 5	737 ± 126	240 ± 65	37 ± 7
AD	4	2/2	76 ± 5	**17.5 ± 11***	**66 ± 14***	835 ± 279	**638 ± 140****	**52 ± 3****

Data are presented as the mean ± SD. Statistically significant differences highlighted in bold were calculated with respect to controls (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

2.4. ELISA

2.4.1. Isolation efficacy

The process of isolation and titres of antibodies were assessed by ELISA as previously described (Hromadkova et al., 2015). Briefly, His-tagged tau 1–441 aa in 0.1 M carbonate buffer (pH 9.5) was applied to a microplate (0.1 μg/50 μl per well) and incubated overnight at 4 °C. After blocking, initial IVIG product (Flebogamma) and AD and control plasma pool, flow-through fractions and eluted fractions were serially diluted as follows 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 with 1% BSA in PBS-T. The experiment was repeated with adjustment of initial Ctrl plasma pool, F-T and eluted fractions to dilutions 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 by 1% BSA in PBS-T. Diluted antibodies (0.1 ml/well) were added to the plate in duplicates and incubated 2 h at RT. The bound nTau antibodies were detected by adding 0.1 ml/well of F(ab´)2-goat anti-human IgG (Fc specific) antibody HRP-conjugate (Novex, Life Technologies, Carlsbad, CA, USA) at dilution 1:10,000 for 30 min at RT followed by washing and a final incubation with 0.1 ml/well of TMB substrate for 30 min at RT in the dark. The non-specific binding of antibodies to empty blocked wells was evaluated for initial and eluted fractions. There was non-specific binding present in the lowest dilution of initial fractions (highest OD 0.254), but it was negligible in the isolated (eluted) antibodies (OD 0.057). The non-specific signal was subtracted from the signal obtained from coated wells.

2.4.2. Reactivity of nTau antibodies against recombinant tau forms

Reactivity of all isolated antibodies against two recombinant forms of tau protein (His-tagged tau 1–441 aa and truncated tau 155–421 aa forms) was measured by ELISA. Tau antigens in 0.1 M carbonate buffer (pH 9.5) were applied to microplate (0.1 μg/50 μl per well) and incubated overnight at 4 °C. BSA in concentration 1 μg/50 μl per well was used as an irrelevant protein to evaluate specificity of isolated antibodies. Then the wells were blocked with 1% BSA in PBS-T for 6 h at 4 °C. Subsequently, the microplate was washed once with 0.1% BSA in PBS-T. The isolated antibodies were diluted as follows nTau-IVIG (1:500), nTau-Ctrl (1:250) and nTau-AD (1:250) with 1% BSA in PBS-T. Diluted antibodies (0.1 ml/well) were added to the coated wells with tau and BSA as well as to empty blocked wells of the plate in duplicates and incubated overnight at 4 °C. The unbound molecules were removed by washing three times with 0.1% BSA in PBS-T. Incubation with 0.1 ml/well of goat anti-human IgG antibody HRP-conjugate at a dilution of 1:10,000 for 30 min at RT was followed by washing the microplate three times as mentioned above. Final incubation was performed for 12 min at RT with 0.1 ml/well of TMB substrate in the dark. The reaction was stopped by 0.1 ml of 1 M H_2SO_4 per well, and the absorbance was measured by Elisa Reader Multiskan® EX (Thermo Scientific, Waltman, MA, USA) at 450 nm and 620 nm as a reference wavelength.

2.4.3. IgG isotyping

Percentages of IgG subclasses were assayed using an IgG Subclass ELISA Kit according to the manufacturer's instructions (Invitrogen, Camarillo, CA, USA).

2.5. Blotting techniques

2.5.1. Western blot immunoassays

Western blot immunoassays were carried out with all four recombinant forms of tau protein and brain-derived proteins obtained from homogenates to compare the reactivity profile of isolated tau-reactive antibodies and anti-tau antibodies with defined epitopes. Antibodies used in Western blots were: isolated natural tau-reactive antibodies (nTau-IVIG, nTau-AD and nTau-Ctrl (1:150) also in their biotin-conjugated forms (1:300)), rabbit polyclonal anti-tau antibody (1:250 (Kristofikova et al., 2014)), Tau 46.1 and Tau 5 antibody (generous gift from Dr. Francisco Garcia-Sierra, 1:60,000 and 1:30,000, respectively), phospho-PHF-tau pThr231 antibody (AT180, Thermo Scientific, Waltman, MA, USA, 1:2000), rabbit polyclonal anti-tau phospho-Ser396 (GenScript, Piscataway, NJ, USA, 1:1000) and detection anti-mouse /anti-rabbit /anti-human IgG antibody HRP-conjugate (Sigma-Aldrich, St. Louis, MO, USA, 1:15,000). All four recombinant His-tagged forms of tau (tau 1–441 aa and tau 155–421 aa in phosphorylated and non-phosphorylated forms, 4 μg of each) were mixed with non-reducing and reducing (2% β-mercaptoethanol) sample loading buffer (Bio-Rad, Hercules, CA, USA) and loaded onto Tricine polyacrylamide gel (10% T, 3% C). In the case of brain homogenates, 25 μg of total protein from supernatants per lane was loaded onto the MINI-PROTEAN® TGX™ 10% precast gels (Bio-Rad, Hercules, CA, USA) and 2 μg of tau 1–441 aa and tau 155–421 aa served as positive controls. Gels were transferred onto nitrocellulose membrane and protein transfer was checked by Ponceau S staining. Membranes were blocked with 10% non-fat dried milk in PBS with 0.1% Tween-20 (PBST) or 5% BSA in PBS-T, antibodies were added in PBS-T with 1% BSA and membranes were incubated overnight at 4 °C. After PBS-T washing steps (five times, each 5 min), membranes were incubated with detection HRP-conjugated IgG antibody or STREP-TACTIN®-HRP Conjugate (Bio-Rad, Hercules, CA, USA, 1:15,000) in PBS-T with 1% BSA for 2 h at RT and subsequently washed with PBS-T (five times, each 5 min). Chemiluminescence signal detection was performed with Clarity™ Western ECL substrate (Bio-Rad, Hercules, CA, USA) and documented by Chemidoc™ XRS + Imaging System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

2.5.2. Determination of avidity index by dot-blot immunoassay

To determine the avidity index of isolated anti-tau antibodies, we applied the dot-ELISA affinity test (Hromadkova et al., 2015) with slight modification. Tau protein (1 μg of tau 1–441 aa or tau 155–421 aa per 100 μl PBS) were spotted onto PVDF membrane and blocked with 5% BSA in PBS-T for 1.5 h at RT. Subsequently, incubation of membranes with isolated tau-reactive antibodies (nTau-IVIG/ − AD/ − Ctrl, 1:150) in PBS-T with 1% BSA followed for 1.5 h at RT. The unbound IgG molecules were removed by washing twice with PBS-T. Then, 5 min incubation with NH_4SCN in PBS (pH 7.0) within a molarity range of 0–2.1 M was included. After washing with PBS-T (twice, 5 min each), membranes were incubated with goat anti-human IgG antibody HRP-conjugate (1:10,000, Sigma-Aldrich, St. Louis, MO, USA) in PBS-T with 1% BSA for 1 h at RT and subsequently washed with PBS-T (three times, 5 min each). The spots were visualized by Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Chemidoc™ XRS + Imaging System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA) was applied for documentation and spot density analysis. All tau samples were spotted in triplicates. The thiocyanate molarity corresponding to 50% reduction in the initial sample signal (referred as avidity index) was calculated from the linear regression equation of logarithmically transformed values of % signal intensity relative to initial signal for each thiocyanate molar concentration (i.e., log ((signal/initial signal) × 100).

3. Results

3.1. Occurrence of natural tau-reactive antibodies in IVIG product Flebogamma and plasma of old cognitively normal individuals versus AD patients

To assess the characteristics of naturally occurring antibodies in the plasma that are reactive to tau protein (nTau antibodies), we simultaneously performed isolation of new batch of nTau antibodies from the IVIG product Flebogamma, the plasma of cognitively normal older controls and AD patients. In this pilot study, we pooled plasma samples from seven cognitively normal older individuals and four AD patients that were established to have low, medium and high plasmatic antibody

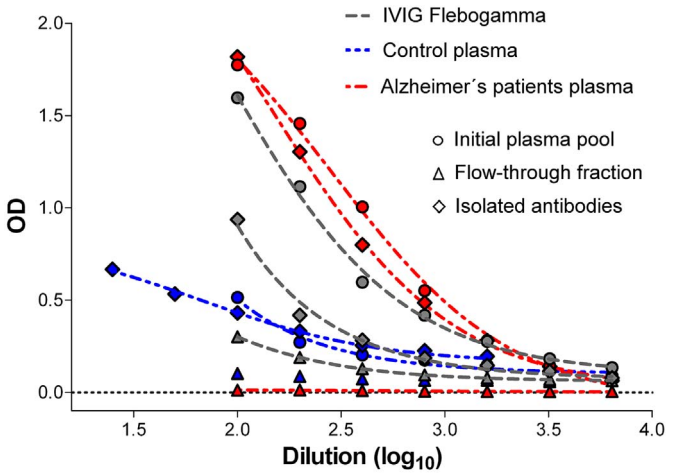


Fig. 1. Isolation efficiency of natural anti-tau antibodies from IVIG, plasma pools of AD patients and cognitively normal older controls estimated by ELISA. Serial dilution of each fraction was probed against His-tagged tau 1–441 aa antigen. The detected signal is expressed as absorbance at 450 nm wavelength depending on the logarithm of samples dilution. His-tagged tau 1–441 aa antigen was coated onto a well at a concentration of 0.1 μg/50 μl in 0.1 M bicarbonate buffer pH 9.5. Initial plasma pool fraction, flow-through fraction (IgG fraction passed through the column with covalently bound His-tagged tau 1–441 aa antigen with no retention), elution fraction containing isolated Tau -reactive antibodies (antibodies fraction retained in the column with covalently bound tau ligand) are illustrated.

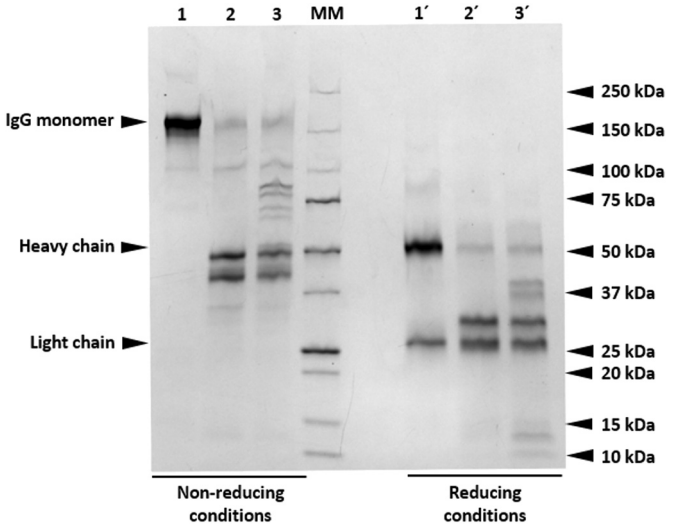


Fig. 2. Verification of purity of isolated antibodies. SDS-PAGE electrophoresis (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio—Rad)) of elution fractions containing natural Tau -reactive antibodies from IVIG Flebogamma product (nTau -IVIG) (1-non-reducing, 1′-reducing conditions), plasma of older cognitively normal controls (nTau -Ctrl) (2, 2′) and AD patients (nTau -AD) (3, 3′) in PBS pH 7.2 followed by Coomassie staining Bio-Safe™ Coomassie Stain (Bio-Rad). Arrowed bands represent 150 kDa band of IgG monomers. Bands below 150 kDa are supposed to be IgG fragments or contaminants.

titers according to ELISA performed with the full-length tau and 155–421 aa tau fragment as antigens (data not shown). The antibodies were purified as described previously (Hromadkova et al., 2015) with newly prepared column. We isolated 8.3 mg of natural tau-reactive antibodies from IVIG product Flebogamma (hereinafter referred to as nTau-IVIG without the intent to generalize for other IVIG products), 3.8 mg of natural AD plasma tau-reactive antibodies (nTau-AD) and 5 mg of antibodies reactive to tau protein from cognitively normal older subjects (nTau-Ctrl), estimated by BCA test. The isolation process was assessed by ELISA in all fractions of isolation (Fig. 1) and purity of isolated antibodies by SDS-PAGE electrophoresis (Fig. 2).

The isolated nTau-Ctrl and nTau-AD antibodies contained impurities or contaminants that represent > 50% of the total protein content. Therefore, the portion of nTau-Ctrl and nTau-AD antibodies was estimated to be approximately 0.4% of total protein amount from the initial pooled sample (Fig. 2). The content of isolated nTau-IVIG antibodies was calculated as 5% of total protein amount from the initial Flebogamma product.

3.2. IgG subclass distribution in the isolated fraction of natural tau-reactive plasma antibodies

We performed an isotyping of IgG subclasses in the isolated tau-reactive antibodies from all three plasma pools. The pools of plasma samples contained the usual distribution of IgG subclasses (data not shown) (Listì et al., 2006; Lock and Unsworth, 2003). We found that the IgG1 and IgG2 subclasses were the most abundant in all three samples of isolated antibodies. The isolated nTau-Ctrl antibodies were threefold enriched with IgG4 subclass in comparison to nTau-AD antibodies and twofold as compared to the nTau-IVIG antibodies. In agreement with our previous finding (Hromadkova et al., 2015), the newly isolated nTau-IVIG antibodies showed enrichment of IgG3 subclass in comparison to the Flebogamma IVIG product. Interestingly, the IgG3 subclass was present in the nTau-AD and nTau-Ctrl antibodies only in trace amount (Fig. 3). We do not attribute the differences in the distribution of IgG subclasses to gender influence because the plasma pool of controls was prepared by mixing samples from four men, and three women and the plasma pool of AD patients contained samples from two men and two women.

3.3. Different reactivity of isolated plasma nTau antibodies against various recombinant forms of tau protein

Firstly, we evaluated the reactivity of isolated plasma antibodies against various recombinant forms of tau protein. All four tau forms were also detected and evaluated using monoclonal and polyclonal anti-tau antibodies with known epitopes. These antibodies revealed and confirmed the upward electrophoretic shift of phosphorylated tau proteins on the gel in comparison with their non-phosphorylated forms (Fig. 4a–c). After optimization of western blot experiments and due to the presence of other proteins than IgG in the isolated nTau-AD and nTau-Ctrl fractions and anti-idiotypic antibodies in the nTau-IVIG

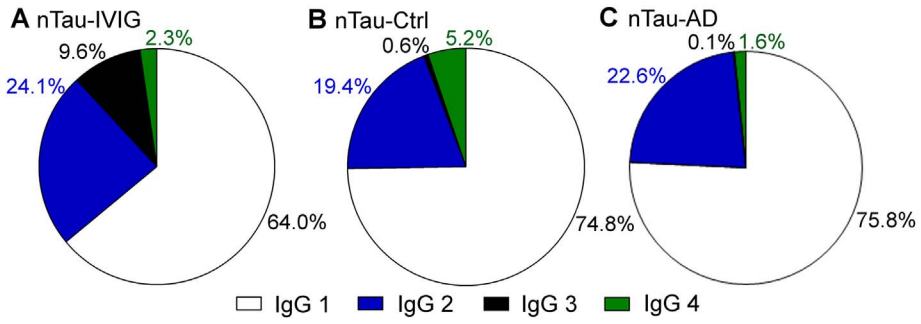


Fig. 3. The distribution of IgG subclasses in isolated antibodies. Isolated antibodies against tau protein from the intravenous immunoglobulin product Flebogamma (a), the pool of plasma samples from seven cognitively normal older subjects (b) and four AD patients (c). Percentages of IgG subclasses were determined by ELISA. The distributions of IgG1 and IgG2 were comparable in all three isolated antibodies with a small deviation in nTau -IVIG. The IgG3 subclass was barely at a detectable level in nTau -AD and nTau -Ctrl in comparison to nTau -IVIG. The isolated antibodies from older individuals showed enrichment of IgG4 subclass threefold in comparison to antibodies from AD patients and twofold as compared to IVIG.

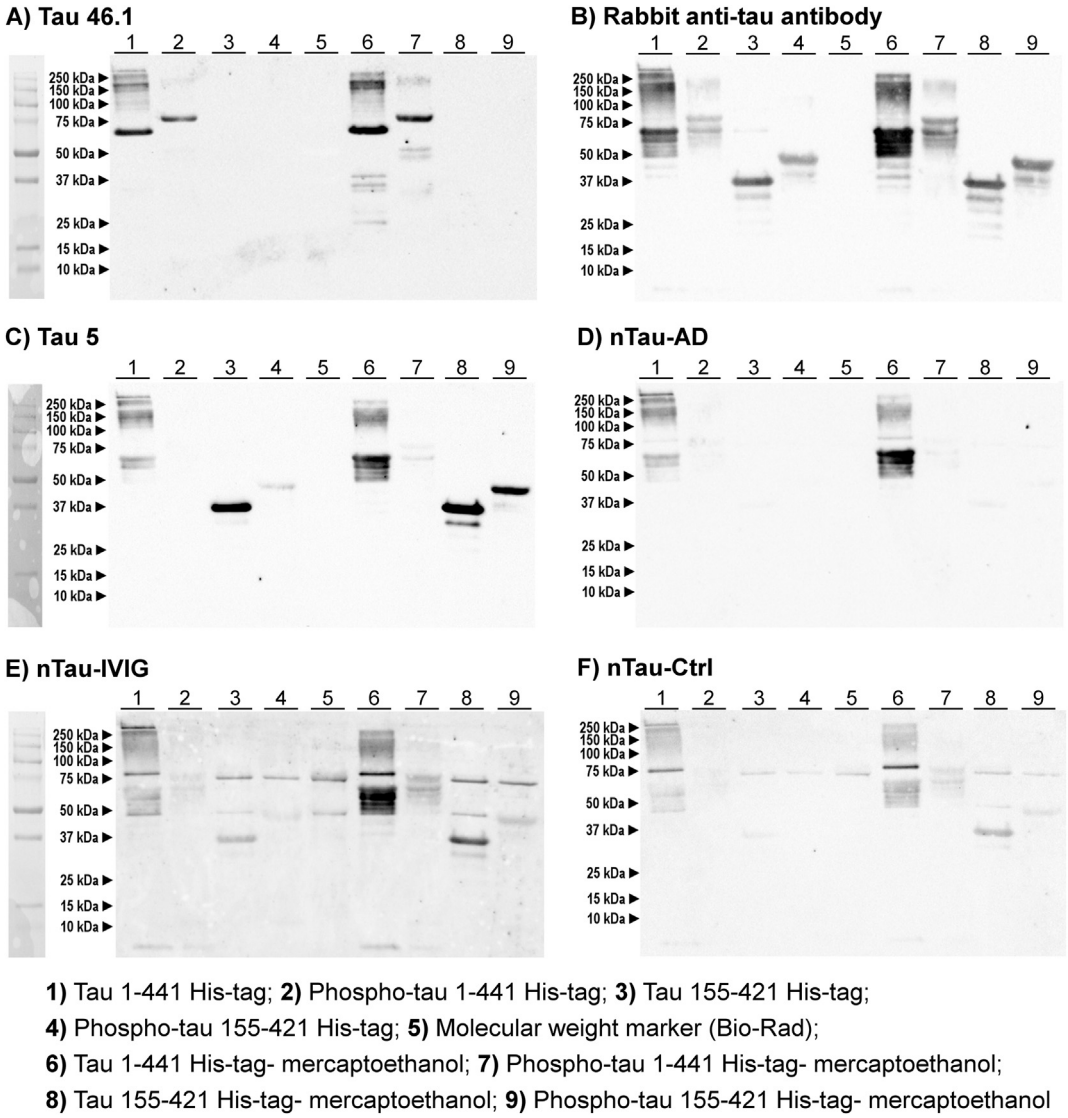


Fig. 4. Reactivity of isolated nTau antibodies against recombinant forms of tau protein on Western blot. The reactivity of isolated nTau antibodies were verified by Western blot against various recombinant His-tagged forms of tau protein in non-phosphorylated (np-) and phosphorylated form (p-) under non-reducing and reducing (− mercaptoethanol) conditions: np-tau 1–441 aa (1), p-tau 1–441 aa (2), np-tau 155–421 aa (3), p-tau 155–421 aa (4), np-tau 1–441 aa - mercaptoethanol (6), p-tau 1–441 aa - mercaptoethanol (7), np-tau 155–421 aa - mercaptoethanol (8) and p-tau 155–421 aa - mercaptoethanol (9). The nitrocellulose membrane was reprobed with monoclonal anti-tau antibodies: Tau 5 (210–241 aa), Tau 46.1 (epitope 428–441 aa) and a polyclonal rabbit anti-tau antibody raised against the tau 1–441 His-Tag antigen (171–194 aa).

fraction, all three isolated antibodies were used at the same initial dilution without previous adjustment to the same concentration. All isolated antibodies fractions applied were less reactive to phosphorylated tau protein forms, but aggregates of tau with higher molecular weight (HMW) were stained (Fig. 4). Recombinant tau protein, both full-length and fragment form aggregates which are partly disintegrated under reducing conditions (Fig. 4). The nTau-IVIG and nTau-Ctrl antibodies reacted with both tau 1–441 aa and tau 155–421 aa and they also produced a strong signal with HMW aggregates. In contrast, nTau-AD antibodies stained only non-phosphorylated tau 1–441 aa both in the monomeric and aggregated form (Fig. 4g).

We also assessed the specificity of isolated nTau antibodies for various forms of tau by ELISA. The results confirmed the findings from Western blot where we observed preference of nTau-AD antibodies towards the tau 1–441 aa as compared to the antibodies isolated from older controls and IVIG (Flebogamma) (Fig. 5).

We evaluated the strength of binding of each isolated nTau antibody with both the full-length (tau 1–441 aa) and truncated (tau 155–421 aa) forms of tau protein by measuring the Avidity index using Dot blot technique. The avidity of nTau-AD antibodies against the His-tagged

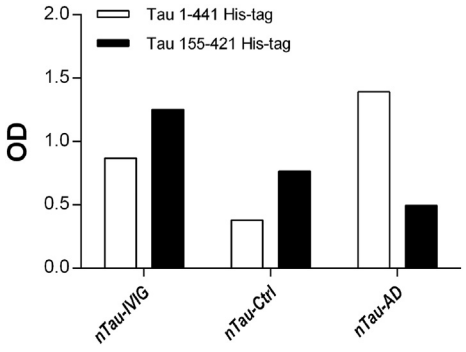


Fig. 5. Reactivity of isolated nTau antibodies against recombinant forms of tau protein in ELISA assay. The specificity of binding of naturally occurring antibodies isolated from IVIG Flebogamma product (nTau -IVIG), plasma of older cognitively normal controls (nTau-Ctrl) and AD patients (nTau -AD) was assessed by ELISA assay against various forms of tau protein. 0.1 μg of tau protein (various forms) were detected with 1:250 of nTau -Ctrl or nTau -AD and 1:500 of nTau -IVIG antibodies.

Table 2
The quantitative data of isolated antibodies reactivity with different tau antigens established by ELISA and Dot-blot immunoassay.

		Isolated natural anti-Tau antibodies from human plasma against	
		Tau 1–441 His-tagged	Tau 155–421 His-tagged
Reactivity ELISA: dilution at OD 0.5[a]	nTau -IVIG Abs	1:195	1:173
	nTau -AD Abs	1:756	1:595
	nTau -Ctrl Abs	1:67	N/A
Avidity index[b] Dot-blots	nTau -IVIG Abs	1.54	1.78
	nTau -AD Abs	0.29	1.50
	nTau -Ctrl Abs	1.27	1.56

[a] The results are expressed as a dilution of antibodies reacting with antigen (0.1 μg/well) to reach OD 0.5 at 450 nm.
[b] Avidity index is expressed as molarity of ammonium thiocyanate (M) that causes the decrease of initial sample signal (in the absence of thiocyanate) on the value of 50%. N/A = not applicable.

tau 1–441 aa form was found to be low (0.29 M of NH_4SCN) in contrast to tau fragment 155–421 aa (1.50 M of NH_4SCN). The nTau-Ctrl and nTau-IVIG antibodies showed comparable high avidity against both forms of tau protein (Table 2). The characteristics of isolated human plasma tau-reactive antibodies are summarized in Table 2.

3.4. Reactivity of plasma nTau antibodies against tau protein forms present in brain tissue

Reactivity of all the isolated plasma nTau antibodies was also verified against native proteins present in the homogenates of human hippocampi obtained from two AD patients and one control subject. The samples were prepared as PBS-soluble protein fractions and SDS-soluble protein fractions from brain homogenate. By Western blot, the samples were probed with well-known monoclonal and polyclonal antibodies specific for different epitopes/forms of tau protein that were found to bind monomeric isoforms of tau in control brain and pathological forms in AD brains (Fig. 6g, h, i, j). According to the reactivity of pSer396 and pThr231 specific antibodies to homogenates of brain tissue from two AD patients (Fig. 6i, h), these two patients were at different stages of the disease. We observed a slight reactivity of pThr231 specific antibody towards recombinant proteins. The antibody cross-reacts with normal native tau but not with the non-phosphorylated recombinant tau protein, thus this reactivity could be attributed to some non-specific signal.

All isolated nTau antibodies reacted similarly with brain-derived proteins from tissue homogenates with a preference towards higher molecular weight (HMW) proteins (Fig. 6a, c and e). We conjugated all three isolated fractions of human antibodies to biotin to avoid added signal from secondary anti-human IgG antibody alone (Fig. 6b, d and f), which we have observed (data not shown). This resulted in an attenuation of the signal against HMW proteins in the isolated antibodies from IVIG Flebogamma and AD plasma and an increased binding of lower molecular forms, especially in the case of nTau-AD antibodies. The biotinylated nTau-AD antibodies showed similar staining pattern as Tau 5 antibody and the rabbit polyclonal anti-tau antibody, which was raised against the recombinant His-tagged tau 1–441 aa (Fig. 6d, g and j). However, the biotinylated nTau-Ctrl antibodies retained the reactivity against the HMW forms and also recognized the lower molecular weight forms of tau protein. The detection of HMW proteins with nTau-Ctrl antibodies was comparable to monoclonal antibody AT180 which has specificity to phospho-Thr231 of tau molecule (Fig. 6f and h).

4. Discussion

Recent studies reported the physiological release of endogenous tau protein from neurons into the interstitial fluid (ISF) which is mediated by neuronal activity (Pooler et al., 2013; Yamada et al., 2014). The secreted or passively released tau could be washed out of ISF through "glymphatic" pathway into the blood (Iliff et al., 2014, 2012) where it can be subjected to fast degradation by proteases and targeted by natural antibodies. As described previously (Bartos et al., 2012; Hromadkova et al., 2015; Klaver et al., 2013; Rosenmann et al., 2006; Smith et al., 2014, 2013), plasma/serum of cognitively healthy individuals and AD patients contains tau-reactive antibodies and their levels are comparable between these groups. To our knowledge, the isolation of tau-reactive antibodies from human plasma samples with subsequent characterization has not been reported previously. We aimed to isolate and characterize the reactivity profile of these systemic naturally occurring antibodies not only by using recombinant tau forms, but also with native forms present in homogenates of brain tissue. We have been able to isolate 8.3 mg of nTau-IVIG antibodies that constitute 5% of the total amount of IgG in initial Flebogamma IVIG product. In contrast, the isolation yield was approximately 0.4% for nTau-AD and nTau-Ctrl antibodies from initial plasma pools when the same isolation procedure and the same 4 mg of full-length tau protein 1–441 aa bound column were used. The reported high content of specific tau-reactive antibodies in the IVIG product Flebogamma is unlikely. We suppose that anti-idiotypic antibodies (Ab2α) (López-Requena et al., 2014) that recognize an idiotype but not a paratope of antigen-specific antibodies, could be isolated as a complex with tau-reactive (antigen-specific) antibodies. Eventually, this complex is dissociated during the acidic elution of antigen-specific antibodies from the column. This assumption is supported by the Western blot with biotinylated nTau-IVIG antibodies in which we observed attenuation of the signal compared to result with non-biotinylated antibodies. In this experimental setup supposedly only antibodies that recognize tau protein can be visualized by streptavidin conjugated to HRP. The fractions of isolated antibodies from pooled plasma samples contained impurities. Although the purification of IgG through protein A or G resin would have given purer fractions, we used the unpurified isolated nTau antibodies for our experiments due to the varying affinity of protein A or G towards different IgG subclasses (Page and Thorpe, 2002), which could skew the consequent IgG isotyping. However, this pilot study should be repeated with the inclusion of more individuals to better reflect the distribution of antibodies in the population of AD patients and age-matched controls.

Interestingly, we have found substantial reactivity of nTau antibodies to a fragment of tau protein 155–421 aa. The strength of binding was confirmed by avidity measurements where we observed higher avidity index of these antibodies (~1.6 M) for tau 155–421 aa fragment as compared to the full-length form. This antigenicity was partly abolished by phosphorylation of this fragment pointing out to epitopes within MTBR domain or related to truncation of tau protein rather than phospho-specificity. This finding corresponds with our previous study (Hromadkova et al., 2015) and study of Rosenmann's group where they observed no significant differences in reactivity of circulating serum IgG against full-length tau and pathologically phosphorylated tau peptide (Rosenmann et al., 2006). However, the isolation procedure was carried out against non-phosphorylated full-length form of tau protein, thus phospho-specific antibodies may not be sorted out. In recent paper, Loeffler et al. showed that Flebogamma IVIG product contains antibodies specific to short peptides of tau protein with phosphorylated sites Ser199 and Ser202 (Loeffler et al., 2015). Additional epitope mapping of these antibodies would provide a better understanding of their reactivity.

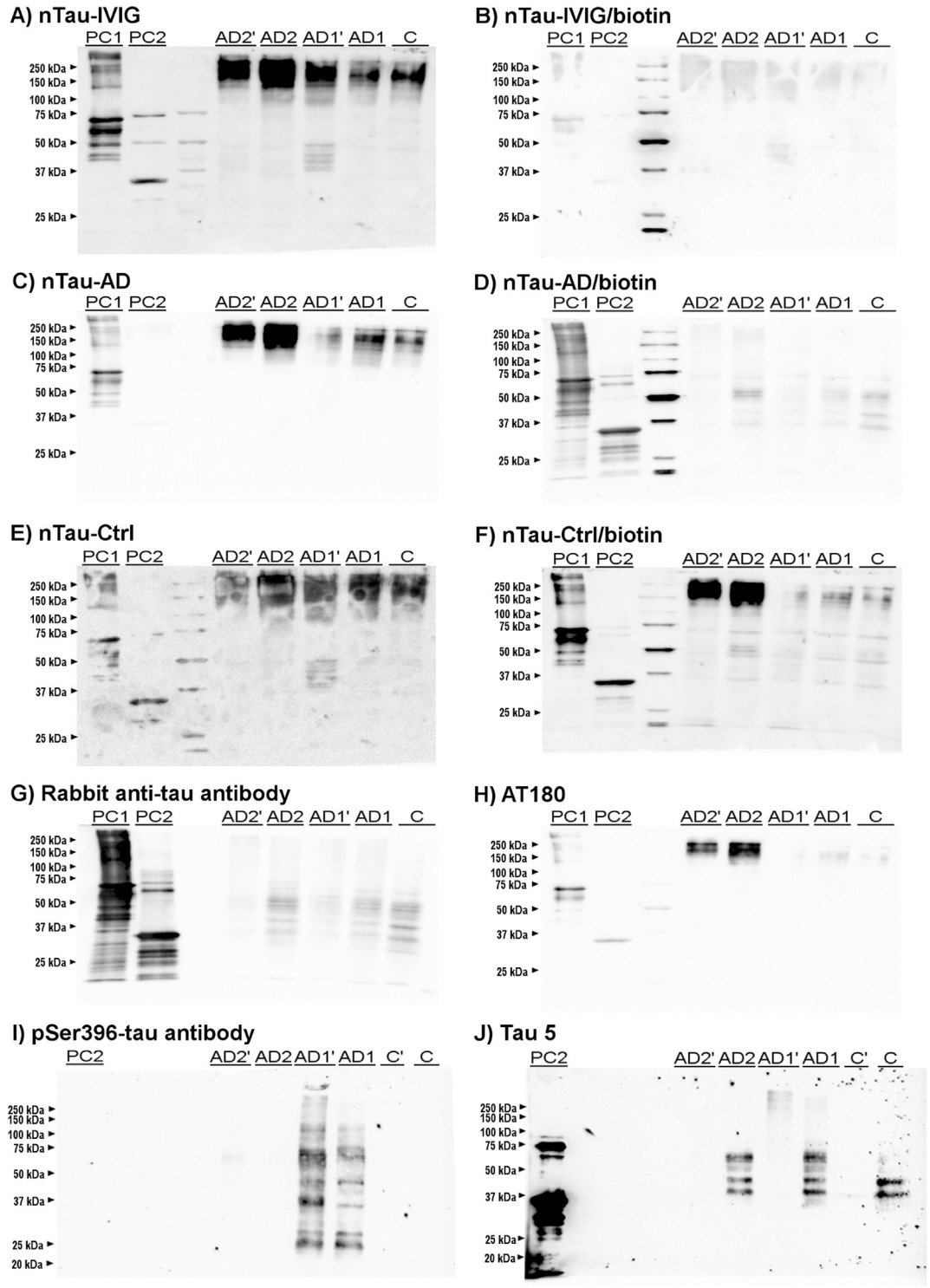


Fig. 6. Reactivity of isolated nTau antibodies against native proteins from brain homogenates. Western blot analysis of human brain homogenates (25 μg of total protein/lane) was carried out using naturally occurring Tau -reactive antibodies isolated from IVIG (nTau -IVIG Abs) product Flebogamma (a), nTau -IVIG Abs conjugated to biotin (b), naturally occurring Tau -reactive antibodies from plasma of AD patients (nTau -AD Abs) (c), nTau -AD Abs conjugated to biotin (d), naturally occurring Tau -reactive antibodies from plasma of older cognitively normal subjects (nTau -Ctrl Abs) (e), nTau -Ctrl Abs conjugated to biotin (f) and rabbit polyclonal anti-tau antibody (g), monoclonal phospho-PHF-tau (pThr231) antibody (AT180, early stage of AD) (h), polyclonal pSer396-tau antibody (late stage of AD) (i) and monoclonal Tau 5 antibody (j). Left hemisphere hippocampi of one control brain and two histopathologically proven AD patient's brain were homogenized in PBS buffer (C- control brains and AD- AD brains) or PBS buffer containing 2% SDS (C'- control brains and AD'- AD brains), respectively. A recombinant fragment of Tau 155–421 with the theoretical molecular weight of 30 kDa (2 μg/well) (PC2) and recombinant full-length form of Tau protein (1–441 aa) appearing on the gel around 75 kDa (PC1) were included as positive controls. The exposure was kept constant for all plasma antibodies and membranes.

To obtain insight into the reactivity of these isolated antibodies with native proteins, we probed brain homogenates of histopathologically diagnosed AD patients and controls with nTau antibodies and other selected polyclonal and monoclonal anti-tau antibodies by Western blot. The most interesting result was the reactivity of nTau antibodies to higher molecular weight forms of tau protein present in the PBS-soluble protein fractions of brain homogenates that were also recognized by the pThr231-tau specific antibody. Its reactivity is described to occur early

during the formation of NFT in the brain, in the structures that are not yet positive for thiazine red (Hawkins et al., 2013; Luna-Muñoz et al., 2007). Moreover, the latest studies have revealed that aggregated oligomeric forms of tau protein may be the most neurotoxic species (Ren and Sahara, 2013; Sahara and Avila, 2014). They can spread tau pathology and are responsible for the cognitive impairment in tauopathy mouse models which can be alleviated by passive immunotherapy specifically targeting tau oligomers (Castillo-Carranza et al., 2014; Yanamandra et al., 2015). However, to rule out both the possible additional signal contributed by the endogenous IgG(s) present in the brain homogenates (Diamond et al., 2013, 2009) and their appearance in the similar molecular weight range on the membrane; the nTau-Abs were biotinylated. The conjugation resulted in different reactivity of nTau-AD and nTau-IVIG antibodies in comparison to antibodies without biotinylation and pointed to the presence of IgG in the homogenates and occurrence of anti-idiotype antibodies in the isolated fraction of antibodies. However, the conjugation of biotin could also interfere with the reactivity of antibody by binding to the lysine residues present on the Fc fragment and in the hinge region of IgG molecules limiting the flexibility of two antigen binding sites (Michaelsen et al., 1977). This effect could be more pronounced at nTau-IVIG antibodies with the content of IgG3 subclass, where the Fc fragment can contribute to their ability to bind antigen (Dillon et al., 2016). On the contrary, the nTau-Ctrl antibodies maintained the reactivity towards HMW forms of tau even after the conjugation (Fig. 6f) possibly also due to the limited lysine content in the hinge region of IgG4 in comparison to other subclasses (Davies and Sutton, 2015).

4.1. Conclusions

Our findings of different reactivity profile of plasma antibodies from cognitively normal individuals and AD patients to different forms of tau protein with the hypothesis of peripheral sink in mind may indicate the participation of adaptive immune system in clearance of the aggregated and truncated tau structures from the brain as was suggested by (Castillo-Carranza et al., 2014) and others (d'Abramo et al., 2013; Yanamandra et al., 2015). This explanation would warrant immunotherapy as a promising approach for the treatment of AD via antibody-associated enhanced clearance of toxic tau aggregates, which is insufficient in AD patients. Although, from our experiments we cannot state whether these antibodies are functioning as opsonization factors for phagocytosis by macrophages or can directly participate in the degradation of formed aggregates. More experiments are also needed to evaluate and characterize these HMW forms.

Competing financial interests

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the project Nr. LO1611 with a financial support from the MEYS under the NPU I program, the Czech Science FoundationP304/12/G069 and by the 260388/SVV/2017 project.

M.K. and L.H. contributed equally to this work, they designed the study, performed all the experiments and prepared the manuscript together. A.B. collected and prepared the human samples and together with Z.B and J.R. reviewed the manuscript.

References

d'Abramo, C., Acker, C.M., Jimenez, H.T., Davies, P., 2013. Tau passive immunotherapy in mutant P301L mice: antibody affinity versus specificity. PLoS One 8, e62402. http://dx.doi.org/10.1371/journal.pone.0062402.

Arai, T., Miklossy, J., Klegeris, A., Guo, J.-P., McGeer, P.L., 2006. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. J. Neuropathol. Exp. Neurol. 65, 19–25.

Balakrishnan, K., Andrei-Selmer, L.-C., Selmer, T., Bacher, M., Dodel, R., 2010. Comparison of intravenous immunoglobulins for naturally occurring autoantibodies against amyloid-beta. J. Alzheimers Dis. 20, 135–143. http://dx.doi.org/10.3233/JAD-2010-1353.

Bartoš, A., Raisová, M., Kopeček, M., 2011. Novelizace české verze Addenbrookského kognitivního testu (ACE-CZ). Cesk Slov Neurol N 74 (107), 681–684.

Bartos, A., Fialová, L., Svarcová, J., Ripova, D., 2012. Patients with Alzheimer disease have elevated intrathecal synthesis of antibodies against tau protein and heavy neurofilament. J. Neuroimmunol. 252, 100–105. http://dx.doi.org/10.1016/j.jneuroim.2012.08.001.

Basurto-Islas, G., Luna-Muñoz, J., Guillozet-Bongaarts, A., Binder, L., Mena, R., García-Sierra, F., 2008. Accumulation of aspartic acid421- and glutamic acid391-cleaved tau in neurofibrillary tangles correlates with progression in Alzheimer disease. J. Neuropathol. Exp. Neurol. 67, 470–483.

Baxter U.S., 2013a. Baxter Announces Topline Results of Phase III Study of Immunoglobulin for Alzheimer's Disease [WWW Document]. URL. http://www.baxter.com/news-media/newsroom/press-releases/2013/05_07_13_gap_study.page (accessed 1.9.17).

Baxter U.S., 2013b. Baxter Presents Additional Data from Phase III Study of Immunoglobulin for Alzheimer's Disease at AAIC [WWW Document]. URL. http://www.baxter.com/news-media/newsroom/press-releases/2013/07_16_13_aaic_gap_data.page (accessed 1.9.17).

Castillo-Carranza, D.L., Sengupta, U., Guerrero-Munoz, M.J., Lasagna-Reeves, C.A., Gerson, J.E., Singh, G., Estes, D.M., Barrett, A.D.T., Dineley, K.T., Jackson, G.R., Kayed, R., 2014. Passive immunization with Tau oligomer monoclonal antibody reverses tauopathy phenotypes without affecting hyperphosphorylated neurofibrillary tangles. J. Neurosci. 34, 4260–4272. http://dx.doi.org/10.1523/JNEUROSCI.3192-13.2014.

Castillo-Carranza, D.L., Guerrero-Muñoz, M.J., Sengupta, U., Hernandez, C., Barrett, A.D.T., Dineley, K., Kayed, R., 2015. Tau immunotherapy modulates both pathological Tau and upstream amyloid pathology in an Alzheimer's disease mouse model. J. Neurosci. 35, 4857–4868. http://dx.doi.org/10.1523/JNEUROSCI.4989-14.2015.

Chesser, A., Pritchard, S., Johnson, G.V.W., 2013. Tau clearance mechanisms and their possible role in the pathogenesis of Alzheimer disease. Front. Neurol. 4. http://dx.doi.org/10.3389/fneur.2013.00122.

Davies, A.M., Sutton, B.J., 2015. Human IgG4: a structural perspective. Immunol. Rev. 268, 139–159. http://dx.doi.org/10.1111/imr.12349.

Davtyan, H., Zagorski, K., Rajapaksha, H., Hovakimyan, A., Davtyan, A., Petrushina, I., Kazarian, K., Cribbs, D.H., Petrovsky, N., Agadjanyan, M.G., Ghochikyan, A., 2016. Alzheimer's disease AdvaxCpG- adjuvanted MultiTEP-based dual and single vaccines induce high-titer antibodies against various forms of Tau and Aβ pathological molecules. Sci Rep 6, 28912. http://dx.doi.org/10.1038/srep28912.

Diamond, B., Huerta, P.T., Mina-Osorio, P., Kowal, C., Volpe, B.T., 2009. Losing your nerves? Maybe it's the antibodies. Nat. Rev. Immunol. 9, 449–456. http://dx.doi.org/10.1038/nri2529.

Diamond, B., Honig, G., Mader, S., Brimberg, L., Volpe, B.T., 2013. Brain-reactive antibodies and disease. Annu. Rev. Immunol. 31, 345–385. http://dx.doi.org/10.1146/annurev-immunol-020711-075041.

Dillon, M.J., Loban, R.A., Reed, D.E., Thorkildson, P., Pflughoeft, K.J., Pandit, S.G., Brett, P.J., Burtnick, M.N., AuCoin, D.P., 2016. Contribution of murine IgG fc regions to antibody binding to the capsule of Burkholderia pseudomallei. Virulence 0. http://dx.doi.org/10.1080/21505594.2016.1176655.

Dodel, R.C., Du, Y., Depboylu, C., Hampel, H., Frölich, L., Haag, A., Hemmeter, U., Paulsen, S., Teipel, S.J., Brettschneider, S., Spottke, A., Nölker, C., Möller, H.J., Wei, X., Farlow, M., Sommer, N., Oertel, W.H., 2004. Intravenous immunoglobulins containing antibodies against beta-amyloid for the treatment of Alzheimer's disease. J. Neurol. Neurosurg. Psychiatry 75, 1472–1474. http://dx.doi.org/10.1136/jnnp.2003.033399.

Dodel, R., Neff, F., Noelker, C., Pul, R., Du, Y., Bacher, M., Oertel, W., 2010. Intravenous immunoglobulins as a treatment for Alzheimer's disease: rationale and current evidence. Drugs 70, 513–528. http://dx.doi.org/10.2165/11533070-000000000-00000.

Dodel, R., Rominger, A., Bartenstein, P., Barkhof, F., Blennow, K., Förster, S., Winter, Y., Bach, J.-P., Popp, J., Alferink, J., Wiltfang, J., Buerger, K., Otto, M., Antuono, P., Jacoby, M., Richter, R., Stevens, J., Melamed, I., Goldstein, J., Haag, S., Wietek, S., Farlow, M., Jessen, F., 2013. Intravenous immunoglobulin for treatment of mild-to-moderate Alzheimer's disease: a phase 2, randomised, double-blind, placebo-controlled, dose-finding trial. Lancet Neurol. 12, 233–243. http://dx.doi.org/10.1016/S1474-4422(13)70014-0.

Fuchs, S., Feferman, T., Meidler, R., Brenner, T., Laub, O., Souroujon, M.C., 2008. The disease-specific arm of the therapeutic effect of intravenous immunoglobulin in autoimmune diseases: experimental autoimmune myasthenia gravis as a model. Isr. Med. Assoc. J. 10, 58–60.

Grifols Biologicals Inc, 2015. A Study to Evaluate Albumin and Immunoglobulin in Alzheimer's Disease [WWW Document]. URL. https://clinicaltrials.gov/show/NCT01561053 (accessed 1.20.17).

Gu, J., Congdon, E.E., Sigurdsson, E.M., 2013. Two novel Tau antibodies targeting the 396/404 region are primarily taken up by neurons and reduce Tau protein pathology. J. Biol. Chem. 288, 33081–33095. http://dx.doi.org/10.1074/jbc.M113.494922.

Hanger, D.P., Wray, S., 2010. Tau cleavage and tau aggregation in neurodegenerative disease. Biochem. Soc. Trans. 38, 1016–1020. http://dx.doi.org/10.1042/BST0381016.

Hawkins, B.E., Krishnamurthy, S., Castillo-Carranza, D.L., Sengupta, U., Prough, D.S., Jackson, G.R., DeWitt, D.S., Kayed, R., 2013. Rapid accumulation of endogenous tau oligomers in a rat model of traumatic brain injury: possible link between traumatic brain injury and sporadic tauopathies. J. Biol. Chem. 288, 17042–17050. http://dx.doi.org/10.1074/jbc.M113.472746.

Hermanson, G.T., 2013. Bioconjugate Techniques. Academic Press.
Hromadkova, L., Kolarova, M., Jankovicova, B., Bartos, A., Ricny, J., Bilkova, Z., Ripova, D., 2015. Identification and characterization of natural antibodies against Tau protein in an intravenous immunoglobulin product. J. Neuroimmunol. 289, 121–129. http://dx.doi.org/10.1016/j.jneuroim.2015.10.017.
Iliff, J.J., Wang, M., Liao, Y., Plogg, B.A., Peng, W., Gundersen, G.A., Benveniste, H., Vates, G.E., Deane, R., Goldman, S.A., Nagelhus, E.A., Nedergaard, M., 2012. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. Sci. Transl. Med. 4, 147ra111. http://dx.doi.org/10.1126/scitranslmed.3003748.
Iliff, J.J., Chen, M.J., Plog, B.A., Zeppenfeld, D.M., Soltero, M., Yang, L., Singh, I., Deane, R., Nedergaard, M., 2014. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. J. Neurosci. 34, 16180–16193. http://dx.doi.org/10.1523/JNEUROSCI.3020-14.2014.
Kayed, R., 2010. Anti-Tau oligomers passive vaccination for the treatment of Alzheimer disease. Hum. Vaccin. 6, 931–935.
Kile, S., Au, W., Parise, C., Rose, K., Donnel, T., Hankins, A., Chan, M., Ghassemi, A., 2015. IVIG treatment of mild cognitive impairment due to Alzheimer's disease: a randomised double-blinded exploratory study of the effect on brain atrophy, cognition and conversion to dementia. J. Neurol. Neurosurg. Psychiatry jnnp-2015-311486. https://doi.org/10.1136/jnnp-2015-311486.
Klaver, A.C., Coffey, M.P., Smith, L.M., Loeffler, D.A., 2013. Comparison of ELISA measurements of anti-Aβ concentrations and percentages of specific binding to Aβ between unfractionated intravenous immunoglobulin products and their purified anti-Aβ antibodies. Immunol. Lett. 154, 7–11. http://dx.doi.org/10.1016/j.imlet.2013.07.008.
Knight, E.M., Gandy, S., 2014. Immunomodulation and AD—down but not out. J. Clin. Immunol. 34 (Suppl. 1), S70–73. http://dx.doi.org/10.1007/s10875-014-0039-y.
Kontsekova, E., Zilka, N., Kovacech, B., Novak, P., Novak, M., 2014. First-in-man tau vaccine targeting structural determinants essential for pathological tau-tau interaction reduces tau oligomerisation and neurofibrillary degeneration in an Alzheimer's disease model. Alzheimers Res. Ther. 6, 44. http://dx.doi.org/10.1186/alzrt278.
Kriştofikova, Z., Fales, E., Majer, E., Klaschka, J., 1995. (3H)Hemicholinium-3 binding sites in postmortem brains of human patients with Alzheimer's disease and multi-infarct dementia. Exp. Gerontol. 30, 125–136. http://dx.doi.org/10.1016/0531-5565(94)00062-X.
Kristofikova, Z., Ricny, J., Kolarova, M., Vyhnalek, M., Hort, J., Laczo, J., Sirova, J., Ripova, D., 2014. Interactions between amyloid-β and tau in cerebrospinal fluid of people with mild cognitive impairment and Alzheimer's disease. J. Alzheimers Dis. 42, S91–98. http://dx.doi.org/10.3233/JAD-132393.
Listì, F., Candore, G., Modica, M.A., Russo, M., Lorenzo, G.D., Esposito-Pellitteri, M., Colonna-Romano, G., Aquino, A., Bulati, M., Lio, D., Franceschi, C., Caruso, C., 2006. A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence. Ann. N. Y. Acad. Sci. 1089, 487–495. http://dx.doi.org/10.1196/annals.1386.013.
Lock, R.J., Unsworth, D.J., 2003. Immunoglobulins and immunoglobulin subclasses in the elderly. Ann. Clin. Biochem. 40, 143–148. http://dx.doi.org/10.1258/000456303763046067.
Loeffler, D.A., 2014. Should development of Alzheimer's disease-specific intravenous immunoglobulin be considered? J. Neuroinflammation 11, 198. http://dx.doi.org/10.1186/s12974-014-0198-z.
Loeffler, D.A., Klaver, A.C., Coffey, M.P., 2015. ELISA measurement of specific antibodies to phosphorylated Tau in intravenous immunoglobulin products. Int. Immunopharmacol. 28, 1108–1112. http://dx.doi.org/10.1016/j.intimp.2015.08.022.
López-Requena, A., Burrone, O.R., Pérez, R., 2014. Anti-idiotypic antibodies. In: Dübel, S., Reichert, J.M. (Eds.), Handbook of Therapeutic Antibodies. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 407–434.
Luna-Muñoz, J., Chávez-Macías, L., García-Sierra, F., Mena, R., 2007. Earliest stages of tau conformational changes are related to the appearance of a sequence of specific phospho-dependent Tau epitopes in Alzheimer's disease. J. Alzheimers Dis. 12, 365–375.
McKhann, G.M., Knopman, D.S., Chertkow, H., Hyman, B.T., Jack, C.R., Kawas, C.H., Klunk, W.E., Koroshetz, W.J., Manly, J.J., Mayeux, R., Mohs, R.C., Morris, J.C., Rossor, M.N., Scheltens, P., Carrillo, M.C., Thies, B., Weintraub, S., Phelps, C.H., 2011. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 7, 263–269. http://dx.doi.org/10.1016/j.jalz.2011.03.005.
Michaelsen, T.E., Frangione, B., Franklin, E.C., 1977. Primary structure of the “hinge” region of human IgG3. Probable quadruplication of a 15-amino acid residue basic unit. J. Biol. Chem. 252, 883–889.
Mioshi, E., Dawson, K., Mitchell, J., Arnold, R., Hodges, J.R., 2006. The Addenbrooke's Cognitive Examination revised (ACE-R): a brief cognitive test battery for dementia screening. Int. J. Geriatr. Psychiatry 21, 1078–1085. http://dx.doi.org/10.1002/gps.1610.
Mirra, S.S., Heyman, A., McKeel, D., Sumi, S.M., Crain, B.J., Brownlee, L.M., Vogel, F.S., Hughes, J.P., van Belle, G., Berg, L., 1991. The consortium to establish a registry for Alzheimer's disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41, 479–486.
Neff, F., Wei, X., Nölker, C., Bacher, M., Du, Y., Dodel, R., 2008. Immunotherapy and naturally occurring autoantibodies in neurodegenerative disorders. Autoimmun. Rev. 7, 501–507. http://dx.doi.org/10.1016/j.autrev.2008.04.010.
Page, M., Thorpe, R., 2002. Purification of IgG using protein A or protein G. In: Walker, J. (Ed.), The Protein Protocols Handbook. Humana Press, pp. 993–994. http://dx.doi.org/10.1385/1-59259-169-8:993.
Pooler, A.M., Phillips, E.C., Lau, D.H.W., Noble, W., Hanger, D.P., 2013. Physiological release of endogenous Tau is stimulated by neuronal activity. EMBO Rep. 14, 389–394. http://dx.doi.org/10.1038/embor.2013.15.
Relkin, N., 2014. Clinical trials of intravenous immunoglobulin for Alzheimer's disease. J. Clin. Immunol. 34 (Suppl. 1), S74–79. http://dx.doi.org/10.1007/s10875-014-0041-4.
Ren, Y., Sahara, N., 2013. Characteristics of tau oligomers. Front. Neurol. 4. http://dx.doi.org/10.3389/fneur.2013.00102.
Rosenmann, H., Meiner, Z., Geylis, V., Abramsky, O., Steinitz, M., 2006. Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. Neurosci. Lett. 410, 90–93. http://dx.doi.org/10.1016/j.neulet.2006.01.072.
Sahara, N., Avila, J., 2014. “Tau oligomers,” what we know and what we don't know. Front. Neurol. 5. http://dx.doi.org/10.3389/fneur.2014.00001.
Schroeder, S.K., Joly-Amado, A., Gordon, M.N., Morgan, D., 2016. Tau -directed immunotherapy: a promising strategy for treating Alzheimer's disease and other tauopathies. J. NeuroImmune Pharmacol. 11, 9–25. http://dx.doi.org/10.1007/s11481-015-9637-6.
Sigurdsson, E.M., 2009. Tau-focused immunotherapy for Alzheimer's disease and related tauopathies. Curr. Alzheimer Res. 6, 446–450.
Smith, L.M., Coffey, M.P., Klaver, A.C., Loeffler, D.A., 2013. Intravenous immunoglobulin products contain specific antibodies to recombinant human tau protein. Int. Immunopharmacol. 16, 424–428. http://dx.doi.org/10.1016/j.intimp.2013.04.034.
Smith, L.M., Coffey, M.P., Loeffler, D.A., 2014. Specific binding of intravenous immunoglobulin products to tau peptide fragments. Int. Immunopharmacol. 21, 279–282. http://dx.doi.org/10.1016/j.intimp.2014.05.009.
Steinitz, M., 2009. Immunotherapy for Alzheimer's disease. Immunotherapy 1, 461–469. http://dx.doi.org/10.2217/imt.09.3.
Yamada, K., Holth, J.K., Liao, F., Stewart, F.R., Mahan, T.E., Jiang, H., Cirrito, J.R., Patel, T.K., Hochgräfe, K., Mandelkow, E.-M., Holtzman, D.M., 2014. Neuronal activity regulates extracellular tau in vivo. J. Exp. Med. 211, 387–393. http://dx.doi.org/10.1084/jem.20131685.
Yanamandra, K., Jiang, H., Mahan, T.E., Maloney, S.E., Wozniak, D.F., Diamond, M.I., Holtzman, D.M., 2015. Anti-tau antibody reduces insoluble tau and decreases brain atrophy. Ann. Clin. Transl. Neurol. 2, 278–288. http://dx.doi.org/10.1002/acn3.176.

Publication IV:

Kinase-loaded magnetic beads for sequential in vitro phosphorylation of peptides and proteins.

Hromadkova, L., Kupcik, R., Vajrychova, M., Prikryl, P., Charvatova, A., Jankovicova, B., Ripova, D., Bilkova, Z., Slovakova, M., Analyst, 2018, 143(2), 466-474.

Stated contributions of the author: Study design. Experimental part (covalent immobilization of enzymes, phosphorylation of low-molecular-weight substrates and tau protein, storage and operational stability of kinase-loaded magnetic beads, blotting techniques). Data analysis. Manuscript design and writing.

Analyst

PAPER

Check for updates

Cite this: *Analyst*, 2018, **143**, 466

Kinase-loaded magnetic beads for sequential *in vitro* phosphorylation of peptides and proteins†

Lenka Hromadkova, [a,b,c] Rudolf Kupcik, [a] Marie Vajrychova, [d,e] Petr Prikryl, [f] Andrea Charvatova, [a] Barbora Jankovicova, [a] Daniela Ripova, [b] Zuzana Bilkova [a] and Marcela Slovakova *[a]

Received 11th September 2017,
Accepted 30th November 2017

DOI: 10.1039/c7an01508a

rsc.li/analyst

Post-translational modifications, including phosphorylation, greatly impact the physiological function of proteins, especially those that are natively unfolded and implicated in many neurodegenerative diseases. However, structural and functional studies of such proteins require fully defined phosphorylation, including those that are not physiological. Thus, the kinases ERK2 and GSK-3β were immobilized to various superparamagnetic beads with carboxylic, aldehyde, Ni^{2+}, or Co^{3+} functional groups, with a view to efficiently phosphorylate peptides and proteins *in vitro*. Full phosphorylation of specific synthetic peptides confirmed that beads were successfully loaded with kinases. Remarkably, enzymes covalently immobilized on carboxylated SeraMag beads remained active upon reuse, with residual activity after 10 uses 99.5 ± 0.34% for GSK-3β and 36.2 ± 2.01% for ERK2. The beads were also used to sequentially phosphorylate recombinant tau, which *in vivo* is a biomarker of Alzheimer's disease. Thus, a system consisting of two fully active kinases immobilized to magnetic beads is demonstrated for the first time. In comparison to soluble enzymes, the beads are easier to handle, reusable, and thus low-cost. Importantly, these beads are also convenient to remove from reactions to minimize contamination of phosphorylated products or to exchange with other kinases.

Introduction

The advantages of immobilized enzymes in comparison to soluble forms cannot be overstated, in light of the massive expansion in the use of the latter in biotechnology. Indeed, immobilized enzymes are now used as recoverable, stable, and specific catalysts in basic biochemical and biotechnological research,[1–4] as well as pharmaceutics.[5] For example, immobilized enzymes are employed to modify or decorate target substrates under controlled conditions to produce bioactive molecules with the desired structure and function.[6] The two-phase structure of such systems also simulates enzyme function *in vivo*, enables studies of the effects of environmental conditions,[7,8] and facilitates substrate and inhibitor profiling *in vitro*.[9,10] Immobilized enzymes can also be separated easily from reactions if necessary, such as in cases where inhibition of enzymatic activity is required prior to subsequent applications. For instance, immobilized kinases can, in theory, be used to phosphorylate recombinant proteins or peptides *in vitro* in a controlled manner, and then purified away to generate phosphorylated products with minimal enzyme contamination compared with those obtained with soluble kinases, which are preferentially used today.[11,12] An enzyme immobilized to magnetic beads removed by magnetic separation can be gently and repeatedly used, which improves the sample handling and economics of such processes.[13,14] We note that phosphorylation is a reversible post-translational modification that significantly impacts the biological activity of many proteins, and is a crucial event in numerous signalling pathways.[15] Therefore, phosphorylation of recombinant proteins may be required to uncover unique biological properties or even achieve function.

In humans, a repertoire of kinases (*i.e.*, the kinome), including the proline-directed kinases GSK-3β (glycogen synthase kinase 3β) and ERK2,[16,17] modulates cellular signalling and many regulatory pathways.[18,19] ERK2 is a cytoplasmic Ser/Thr kinase ubiquitously expressed, especially in the central nervous system.[20] Indeed, a growing body of evidence indicates

[a]*Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice 532 10, Czech Republic. E-mail: Marcela.Slovakova@upce.cz*
[b]*National Institute of Mental Health, Klecany 250 67, Czech Republic*
[c]*Department of Physiology, Faculty of Science, Charles University in Prague, Prague 128 44, Czech Republic*
[d]*Biomedical Research Center, University Hospital Hradec Kralove, Hradec Kralove 500 05, Czech Republic*
[e]*Department of Molecular Pathology and Biology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove 500 01, Czech Republic*
[f]*Institute of Pathological Physiology, First Faculty of Medicine, Charles University in Prague, Prague 128 53, Czech Republic*
†Electronic supplementary information (ESI) available. See DOI: 10.1039/c7an01508a

This journal is © The Royal Society of Chemistry 2018

that ERK2 phosphorylates clinically relevant biomarkers.[21,22] Similarly, GSK-3β is a Ser/Thr kinase expressed in all mammalian tissues, and abundantly so in the developing brain.[23] GSK-3β kinase strongly prefers pre-phosphorylated substrates with motif SXXXpS, where S is a serine, X is any residue, and pS is a phosphorylated serine.[24,25] Of note, both kinases facilitate the production of various phosphorylated forms of tau, a potential biomarker of Alzheimer's disease, as reviewed in ref. 26. Moreover, tau pre-phosphorylated by ERK2 is a more favourable substrate for subsequent phosphorylation by GSK-3β.[27,28]

We now describe the selection of an optimal strategy to immobilize ERK2 and GSK-3β on magnetic beads to generate reusable biocatalysts of protein phosphorylation. To our best knowledge, only affinity immobilization of recombinant GST-tagged GSK-3β on GSH-functionalized magnetic beads has been attempted to date.[10] On the other hand, we report for the first time that covalent immobilization of ERK2 and GSK-3β produces highly reusable, stable kinases. These immobilized systems were tested against recombinant tau, a clinically relevant model protein, to obtain sequentially phosphorylated products of high purity, as assessed by western blotting and mass spectrometry.

Experimental section

Reagents and chemicals

Recombinant, active, His-tagged rabbit GSK-3β (19 841 U mg^{-1}) was purchased from Sigma-Aldrich (St Louis, MO, USA). Active rabbit muscle GSK-3β (5 000 000 U mg^{-1}), isolated from *E. coli* overexpressing it, was obtained from New England Biolabs (Ipswich, MA, USA). Recombinant, active human ERK2/MAPK1 (401 000 U mg^{-1}), hereafter referred to as ERK2, was produced by Biaffin GmbH & Co KG (Kassel, Germany). Nonporous superparamagnetic SiMAG-IDA and SiMAG-IDA/Ni^{2+} particles (1 μm diameter, number of functional groups unknown) were purchased from Chemicell GmbH (Berlin, Germany), while superparamagnetic aldehyde-modified BcMag beads (1 μm diameter, ~210 μmol aldehyde per g, ~40 EMU g^{-1}) were obtained from BioClone Inc. (San Diego, CA, USA). Carboxylated SeraMag SpeedBeads (superparamagnetic, 0.816 μm diameter, ~507 μmol carboxylate per g, ~25 EMU g^{-1}) were procured from Thermo Scientific (Fremont, CA, USA). Magnetic suspension agitations and separations were performed using a Grant Bio PTR-30 rotator (Wolf-Laboratories, Pocklington, York, UK) and a Dynal MPC-S magnetic separator (Biotech, CZ). Low molecular-weight substrates, namely phosphopeptide CREB (1796.0 Da, sequence KRREILSRRPpSYR), ε-eIF2B (1991.0 Da, sequence RRRAAEELDSRAGpSPQL), and synthetic TH 24-33 (1085.6 Da, sequence KQAEAVTSPR) were obtained from New England Biolabs (Ipswich, MA, USA), Enzo Life Sciences (Farmingdale, NY, USA), and Calbiochem (San Diego, CA, USA), respectively. Tau 46.1, an antibody to tau (1 : 60 000) was a generous gift from Dr F. Garcia-Sierra (Mexico), while AT180, an antibody to tau phosphorylated at T231 (1 : 2000) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies to tau phosphorylated at S356 (1 : 2000) and S396 (1 : 2000) were obtained from Assay Biotech (Sunnyvale, CA, USA) and GenScript (Piscataway, NJ, USA). Anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1 : 15 000) were obtained from Sigma-Aldrich.

Immobilization of kinases to magnetic beads

Oriented immobilization of His-tagged GSK-3β. His-tagged GSK-3β was immobilized by affinity to nonporous magnetic SiMAG-IDA particles decorated with Ni^{2+} and Co^{3+}. The enzyme was immobilized on SiMAG-IDA/Ni^{2+} beads according to ref. 29, with slight modification. Briefly, 50 U (52.1 pmol) of the enzyme was mixed for 30 min at room temperature with 0.5 mg washed beads in 0.1 M HEPES buffer pH 8.0 and 0.5 M NaCl. The beads were then washed three times in the same buffer. Similarly, the enzyme was bound to magnetic SiMAG-IDA/Co^{3+} beads according to ref. 30 and 31. In brief, 0.5 mg SiMAG-IDA particles were washed with ultrapure water and charged for 3 min at room temperature with 1 mL 0.2 M $CoCl_2$. After washing with ultrapure water and 0.1 M phosphate buffer pH 7, the beads were incubated for 90 min at room temperature with 1 mL 0.05% hydrogen peroxide prepared in the same buffer, washed with buffer, incubated overnight at 4 °C with 50 U (52.1 pmol) His-tagged GSK-3β in buffer, and washed another three times with buffer.

Non-oriented covalent immobilization of GSK-3β and ERK2. Untagged recombinant kinases were immobilized *via* free amine groups to aldehyde-modified BcMag beads and carboxylated SeraMag particles. Briefly, 0.6 mg BcMag-aldehyde beads were washed with 0.1 M phosphate buffer pH 7, and incubated overnight at room temperature with 5000 U (21.3 pmol) GSK-3β in the same buffer, but supplemented with 24 mM $NaCNBH_3$ to reduce Schiff bases[32] formed between terminal autoreactive aldehydes in beads and free amines in proteins. The beads were then washed five times with buffer. On the other hand, EDC/sulfo-NHS chemistry[33] was applied to covalently immobilize kinases onto carboxylated SeraMag particles. In brief, 0.6 mg beads were washed in 0.05 M MES buffer pH 6, activated for 30 min at room temperature with 120 mM EDC and 20 mM sulfo-NHS in the same buffer, and washed another time with buffer. The beads were then incubated overnight at 4 °C with 5000 U (21.3 pmol) GSK-3β or 822 U (47.9 pmol) ERK2 in buffer, washed three times with 0.05 M MES buffer pH 6, and then three times with 0.04 M HEPES buffer pH 7.2.

Phosphorylation of low-molecular weight substrates

Phosphorylation of substrates with SXXXpS motif by GSK-3β. Soluble or immobilized GSK-3β was assayed for 5 or 6 h at 30 °C against 1 μg of the low-molecular weight phosphopeptides CREB and ε-eIF2B in 50 μL (for soluble kinase) or 200 μL (for 0.2 mg beads) of Reaction Buffer I (NEBuffer™ for Protein Kinases purchased from New England BioLabs) or in-house Reaction Buffer II, which consists of 0.04 M HEPES pH

7.2, 5 mM $MgCl_2$, 2 mM DTT, and 5 mM EGTA. All reactions were supplemented with 1 mM ATP (New England Biolabs). Similarly, 1 μg of low molecular-weight substrates in 50 μL was phosphorylated with the supernatant collected after immobilization to investigate residual unbound GSK-3β. Phosphorylation was assessed by mass spectrometry, and reactions without GSK-3β were used as negative control. To evaluate stability, SeraMag loaded with GSK-3β was assayed for 6 h at 30 °C against 1 μg of ε-eIF2B in Reaction Buffer I and 1 mM ATP, washed three times with 40 mM HEPES buffer pH 7.2, and stored at 4 °C until reused in a subsequent reaction.

Phosphorylation of low-molecular weight substrates by ERK2. ERK2 was assayed according to ref. 34, with slight modification. Briefly, the enzyme, soluble or immobilized to SeraMag beads, was incubated for 6 h at 30 °C with 1 μg TH 24-33 in 50 μL (for soluble kinase) or 200 μL (for 0.2 mg beads) of Reaction Buffer I or II supplemented with 1 mM ATP. Supernatants collected after immobilization were also assayed against 1 μg TH 24-33 in 50 μL Reaction Buffer I to investigate residual unbound ERK2. In all cases, phosphorylation was examined by mass spectrometry, and substrates incubated without ERK2 or reacted with heat-inactivated ERK2 were used as negative control.

Operational stability of kinase-loaded SeraMag beads

To characterize operational stability, SeraMag beads loaded with ERK2 or GSK-3β were assayed twice daily, 12 h apart, over five days. In brief, 0.2 mg enzyme-loaded was incubated at 30 °C with 1 μg of the corresponding low molecular-weight substrate in Reaction Buffer II with 1 mM ATP. Beads were washed three times between assays with 40 mM HEPES buffer pH 7.2.

Sequential phosphorylation of recombinant tau

SeraMag beads (0.4 mg) loaded with ERK2 were washed five times with 40 mM HEPES buffer pH 7.2, and reacted for 20 h at 30 °C with 5 μg recombinant tau isoform 2N4R (amino acids 1–441, rPeptide, Bogart, GA, USA) in 0.2 mL Reaction Buffer II and 1 mM ATP. Subsequently, the phosphorylated product was incubated for 20 h at 30 °C in 0.2 mL Reaction Buffer II, 1 mM ATP, and 0.4 mg SeraMag beads loaded with GSK-3β and pre-washed in 40 mM HEPES buffer pH 7.2. The final product was analysed by western blotting[35] and by mass spectrometry.

Preparation of tau phosphopeptides

Digestion of phosphorylated tau. Recombinant tau phosphorylated in Reaction Buffer I with 1 mM ATP was incubated for 60 min at 37 °C with 25 mM DTT in 50 mM NH_4HCO_3, and reacted for 30 min at room temperature in the dark with 50 mM iodoacetamide in 50 mM NH_4HCO_3. The reaction was quenched by adding DTT in 50 mM NH_4HCO_3 to a final concentration of 50 mM, and incubating for 15 min at room temperature. Finally, the mixture was digested overnight at 37 °C with 1 : 50 w : w sequencing-grade trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. Digestion was terminated by adding 5% trifluoroacetic acid to pH 2–3.

Enrichment of using TiO_2. Peptides from phosphorylated tau were enriched in batch mode using 1 mg TiO_2 particles (10 μm, Titanspehere™ TiO Bulk Material, GL Sciences, Japan), which was equilibrated with 200 μL of 80% acetonitrile/0.1% trifluoroacetic acid, and then with 200 μL of 80% acetonitrile/5% trifluoroacetic acid/1 M lactic acid digested phosphorylated tau was then suspended in the latter, and mixed with particles for 1 h. Subsequently, particles were washed twice with 80% acetonitrile/5% trifluoroacetic acid/1 M lactic acid, twice with 80% acetonitrile/0.1% trifluoroacetic acid, and once with 20% acetonitrile/0.5% trifluoroacetic acid. Phosphorylated peptides were then eluted with 1% ammonia over 15 min, acidified with 5% trifluoroacetic acid to pH 2–3, and stored until analysis.

Mass spectrometry

Samples were prepared according to standard protocols with slight modification, and analysed on MALDI-LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA), Autoflex II MALDI-TOF/TOF (Bruker Daltonics, Billerica, MA, USA), or 4800 MALDI/TOF/TOF Analyzer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

Desalting. For analysis on MALDI-LTQ Orbitrap XL, reversed-phase microcolumns for desalting and concentration were prepared in-house using GELoader tips (Eppendorf, Hamburg, Germany) and POROS Oligo R3 (Life Technologies, Carlsbad, CA, USA) as previously described.[36] Briefly, peptides were loaded using gentle air pressure to pre-washed and pre-wetted microcolumns, which were then washed with 15 μl 0.1% trifluoroacetic acid. Subsequently, peptides were eluted directly onto the MALDI target with 0.75 μl of 10 mg mL^{-1} DHB matrix, allowed to dry, and analysed. For analysis on 4800 MALDI/TOF/TOF Analyzer, samples were also desalted with the same microcolumns, but eluted into a vial using 10 μL 60% acetonitrile/0.1% trifluoroacetic acid, mixed with an equal volume of 5 mg mL^{-1} DHB matrix, spotted onto the MALDI sample plate, allowed to dry, and analysed. For analysis on Autoflex II MALDI-TOF/TOF, samples were desalted using ZipTip microtips from Millipore (Bedford, MA, USA). Eluates were mixed with an equal volume of 20 mg mL^{-1} DHB matrix, spotted onto the MALDI sample plate, left to dry, and analysed. For all samples, the DHB matrix was prepared in 50% acetonitrile/0.1% trifluoroacetic acid/1% H_3PO_4 v/v/v.

MALDI-LTQ Orbitrap mass spectrometry after simple chromatography. To detect a higher number of phosphorylated peptides, samples of digested tau were separated prior to mass spectrometry using a home-made RP microcolumn (length 25 mm, internal diameter 250 μm) packed with 2.7 μm Ascentis® Express Peptide ES-C18 particles (Sigma-Aldrich, St Louis, MO, USA). These microcolumns were fitted in an FEP tubing (length 40 mm, outer diameter 1/16″, internal diameter 0.25 mm) and terminated with a short piece of fused silica capillary (360 μm outer diameter, 50 μm inner diameter) in one end, as previously described.[37] A 25 μl gas-tight micro-

 This journal is © The Royal Society of Chemistry 2018

syringe (Hamilton, Reno, NV, USA) was used to load solvents and samples to a column that had been washed with 20 μL 80% acetonitrile/0.1% trifluoroacetic acid and equilibrated with 20 μl 2% acetonitrile/0.1% trifluoroacetic acid v/v. After loading, peptides were eluted by a non-linear gradient of acetonitrile created by aspiration of 4 μl each of a series of solvents containing 0.1% trifluoroacetic acid and a decreasing concentration of acetonitrile (32%, 26%, 20%, 14%, 8% and 2%) into the syringe. Eluates were directly spotted onto 24 spots on the MALDI sample plate and covered with 10 mg ml^{-1} DHB matrix prepared as described, dried, and analysed on MALDI-LTQ Orbitrap XL.

Data acquisition and evaluation. Prepared samples were analysed on MALDI-LTQ Orbitrap XL in positive mode with resolution 60 000 FWHM at *m*/*z* 400. Spectra were processed in mMass, an open-source software (http://www.mmass.org), and mapped using MS-Fit, a component of ProteinProspector v5.18.1 (http://prospector.ucsf.edu/prospector/mshome.htm), with mass tolerance 5 ppm. Microtubule-associated tau isoform 2 (Pubmed Protein accession no. 6754638) was used as reference, and results were manually validated by searching for neutral losses of phosphoric acid(s), and by direct comparison of peptides from phosphorylated and unphosphorylated tau.

Semi-quantitative evaluation of phosphorylation rate. For each low-molecular weight substrate, the relative intensities of unphosphorylated and phosphorylated forms were extracted from mass spectra, summed, and set as 100%, to which the proportion of unphosphorylated and phosphorylated forms was normalized.[38] Experiments were performed at least thrice, and data are reported as mean ± SD. The phosphorylated and unphosphorylated forms of CREB, a substrate of GSK-3β, have *m*/*z* 1796.98 and 1876.94, respectively. For ε-eIF2B, another GSK-3β substrate, these forms have *m*/*z* 1991.98 and 2071.94, respectively. For TH 24-33, a substrate of ERK2, the unphosphorylated form has *m*/*z* 1086.59, while the phosphorylated form as *m*/*z* 1166.56. The analysis of variance (ANOVA) was performed to determine operational and storage stability of ERK2-loaded and GSK-3β-loaded magnetic SeraMag beads expressed as residual activity (%). The statistical analysis was performed in R software for statistical computing,[39] version 3.1.1. The significance level was set at <0.05.

Results and discussion

Kinase immobilization and activity against peptide substrates

Immobilized enzymes have been investigated with considerable interest due to several advantages over soluble forms or alternative technologies. Such advantages include reusability, higher stability, low cost, easy separation by magnet and high final product purity, although these also depend on the type of solid phase used, the enzyme, and the immobilization strategy.[40] To obtain functionally active magnetic beads that phosphorylate peptides and proteins with all of these advantages, the proline-directed kinases ERK2 and GSK-3β were immobilized to various superparamagnetic particles. Immobilization was achieved either by affinity to beads decorated with Ni^{2+} or Co^{3+}, or by covalent bonding of amine groups in proteins to beads functionalized with carboxylic or aldehyde groups.

Kinase-specific low molecular-weight substrates were used to test activity following immobilization. In particular, phosphopeptides corresponding to CREB (KRREILSRRPpSYR, 1796.0 Da) and ε-eIF2B (RRRAAEELDSRAGpSPQL, 1991.0 Da) were used to assay GSK-3β, which phosphorylates the first serine in the phosphopeptide sequence SXXXpS. On the other hand, a peptide corresponding to tyrosine hydroxylase amino acids 24-33, sequence KQAEAVTSPR (1085.6 Da), was used to test ERK2, which phosphorylates S31.

Since only pmols of kinase was immobilized per mg of magnetic beads, conventional analytical methods such as bicinchoninic acid assay are not suitable to determine the amount of enzyme bound to magnetic beads. Thus, we emphasize that phosphorylation rates calculated from mass spectrometry, as described in Experimental section, are mainly semi-quantitative. Nevertheless, we found that nearly 100% kinase activity was retained immediately following immobilization, as summarized in Table 1. Notably, little or no residual unbound kinase activity was detected in supernatants after immobilization (Fig. 1, 2 and 4), suggesting that nearly all pro-

Table 1 Kinase activity immediately after immobilization to magnetic particles, as measured against specific low-molecular weight substrates

Recombinant kinase	Magnetic particles	Enzyme bound[a]	Phosphorylation conditions	Phosphorylation rate[b] [%]
His-tagged GSK-3β, rabbit, 19 841 U mg^{-1} (Sigma-Aldrich)	SiMAG-IDA/Ni^{2+}, number of functional groups unknown (Chemicell GmbH)	20 U (20.84 pmol/ 0.2 mg beads)	5 h, 30 °C	~90.0
His-tagged GSK-3β, rabbit, 19 841 U mg^{-1} (Sigma-Aldrich)	SiMAG-IDA, number of functional groups unknown (Chemicell GmbH), Co^{3+} modified	20 U (20.84 pmol/ 0.2 mg beads)	5 h, 30 °C	~90.0
GSK-3β, rabbit, 5 000 000 U mg^{-1} (New England Biolabs)	Aldehyde-modified BcMag beads, ~210 μmol aldehyde per g (BioClone Inc.)	1667 U (7.1 pmol/ 0.2 mg beads)	5 h, 30 °C	~95.0
GSK-3β, rabbit, 5 000 000 U mg^{-1} (New England Biolabs)	Carboxylate-modified SeraMag SpeedBeads, ~507 μmol carboxyl per g (Thermo Scientific)	1667 U (7.1 pmol/ 0.2 mg beads)	6 h, 30 °C	96.6 ± 2.79
ERK2/MAPK1, human, 401 000 U mg^{-1} (Biaffin GmbH & Co KG)		275 U (15.97 pmol/ 0.2 mg beads)	6 h, 30 °C	91.9 ± 3.78

[a] Same as the amount of enzyme added to beads, since no kinase activity was detected in the supernatant collected after immobilization. [b] Calculated according to ref. 38 as described in Experimental section, and is reported as mean ± SD.

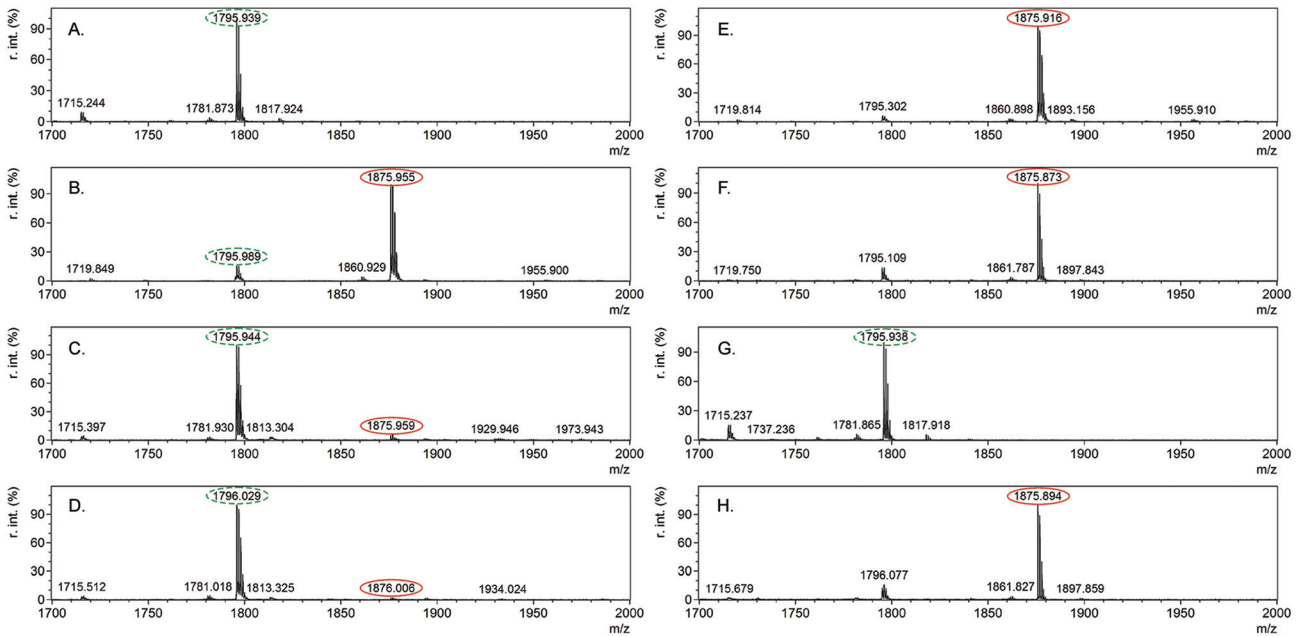


Fig. 1 Representative mass spectra of unphosphorylated and phosphorylated CREB, a specific low-molecular weight substrate of GSK-3β. (A) Unphosphorylated CREB. CREB was then phosphorylated for 5 h at 30 °C in Reaction Buffer I containing (B) 0.2 mg SiMAG Ni^{2+} beads loaded with GSK-3β, (C) supernatant collected after immobilization of GSK-3β to Ni^{2+} beads, (D) 0.2 mg enzyme-loaded Ni^{2+} beads stored for six days at 4 °C, (E) soluble His-tagged GSK-3β, (F) 0.2 mg Co^{3+} beads loaded with GSK-3β, (G) supernatant collected after binding GSK-3β to Co^{3+} beads, and (H) 0.2 mg of enzyme-loaded Co^{3+} beads stored for six days at 4 °C. Unphosphorylated peptides are marked with green dashed circles, while phosphorylated forms are circled in red. All spectra were obtained on Autoflex II MALDI-TOF/TOF.

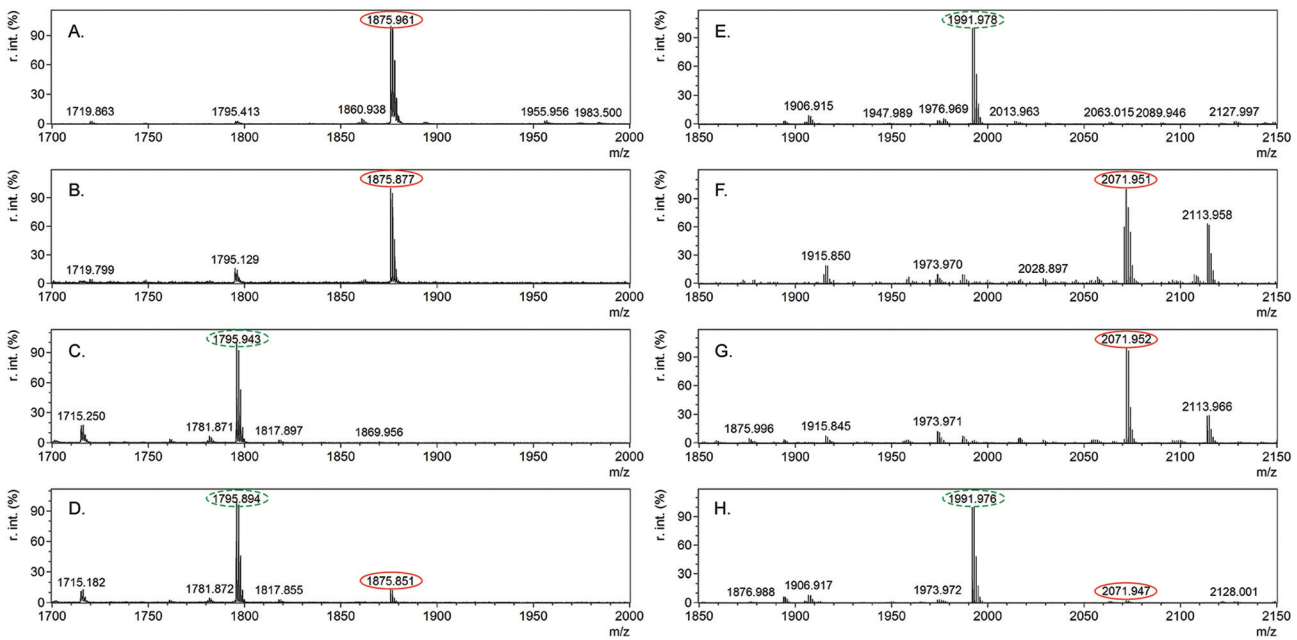


Fig. 2 Representative mass spectra of unphosphorylated and phosphorylated CREB and ε-ILF2, which are specific low-molecular weight substrates of GSK-3β. CREB was phosphorylated for 5 h at 30 °C in Reaction Buffer I containing (A) soluble GSK-3β, (B) 0.2 mg BcMag beads loaded with GSK-3β, (C) supernatant collected after loading GSK-3β to BcMag beads, and (D) 0.2 mg enzyme-loaded BcMag beads stored for six days at 4 °C. (E) Unphosphorylated ε-elF2B. ε-elF2B was also phosphorylated for 6 h at 30 °C by (F) soluble GSK-3β, (G) 0.2 mg enzyme-loaded SeraMag beads, and (H) supernatant collected after immobilizing GSK-3β to SeraMag beads. Unphosphorylated CREB and ε-elF2B are marked with green dashed circles, while phosphorylated forms are circled in red. Spectra A–D were obtained on Autoflex II MALDI-TOF/TOF and E–H were obtained on MALDI-LTQ Orbitrap XL.

This journal is © The Royal Society of Chemistry 2018

teins were successfully immobilized. We note that the amount of functional groups on magnetic beads, when known, was generally in excess compared to the amount of kinase loaded (Table 1). Taken together, we conclude that kinases were successfully immobilized with negligible loss of function, and fully phosphorylate kinase-specific substrates under conditions described in Experimental section.

Affinity immobilization of His-tagged kinases

To our knowledge, GSK-3β has been immobilized only *via* affinity between a GST tag and glutathione-modified magnetic beads.[10] Indeed, since site-specific immobilization *via* a protein tag would likely affect enzyme activity only minimally, we first attempted to immobilize recombinant kinases with a histidine anchor, which confers strong affinity to SiMAG-IDA magnetic particles charged with bivalent metal ions such as Ni^{2+} or Co^{2+}.[41] However, to avoid direct contact between the kinase and an oxidizing agent and thereby increase stability, beads charged with Co^{2+} were first oxidized to Co^{3+} prior to kinase immobilization.[31] Co^{3+} forms exchange-inert complexes with bound proteins to generate a stable "irreversible" linkage.[30]

Immobilized His-tagged GSK-3β was evaluated against a CREB peptide, using mass spectra of unphosphorylated peptides and peptides phosphorylated by soluble His-tagged GSK-3β as controls (Fig. 1A+E). Complete phosphorylation of the substrate was achieved from Ni^{2+} and Co^{2+} beads immediately after loading with His-tagged GSK-3β (Fig. 1B+F and Table 1). Kinase activity was not detected or detected only in trace amount (less than 5% of phosphorylated substrate) in the supernatant recovered after binding, confirming that the enzyme was captured quantitatively (Fig. 1C+G). However, Ni^{2+} beads loaded with enzyme lost kinase activity after six days at 4 °C (Fig. 1D), while His-tagged GSK-3β immobilized *via* Co^{3+} was stable (Fig. 1H).

Of note, site-specific immobilization *via* protein tags was previously reported to enhance the stability and catalytic activity of several enzymes.[42,43] Thus, His-tagged ERK2 was immobilized on SiMAG-IDA/Co^{3+} magnetic particles for the first time, as described in ESI (Method S1†). However, immobilization did not enhance phosphorylation of TH 24-33, a specific substrate peptide, in comparison to soluble His-tagged ERK2, even after reactions were extended to 16 h (ESI, Method S1, Fig. S2A+B†).

Covalent immobilization

To the best of our knowledge, covalent immobilization of GSK-3β and ERK2 to magnetic particles without loss of activity has not been reported previously. Thus, we attempted to covalently attach these enzymes to two types of magnetic beads *via* free primary amines, as described in Experimental section. The enzymes were then assayed after immobilization against specific low-molecular weight substrates as described. For GSK-3β, unphosphorylated CREB and ε-eIF2B peptides were used as control, along with peptides phosphorylated by soluble GSK-3β (Fig. 2A+E+F).

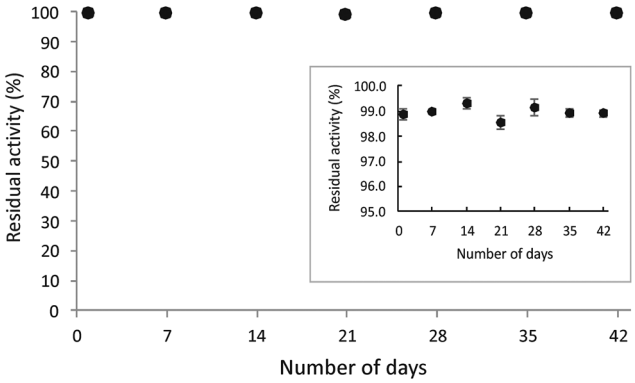


Fig. 3 Stability of SeraMag beads loaded with GSK-3β and stored at 4 °C. Kinase activity was evaluated in terms of the relative intensities of peaks with *m*/*z* 1991.98 (unphosphorylated ε-eIF2B) and 2071.94 (phosphorylated ε-eIF2B). These intensities were summed and set as 100%, to which the proportion of phosphorylated substrates was normalized. Data were collected in triplicate, and are reported as mean ± SD. There was no statistically significant change of GSK3 enzyme activity during the storage as determined by one-way ANOVA (p = 0.8021). Inset: Axis *y* zoomed.

GSK-3β was immobilized onto aldehyde-modified BcMag beads by $NaCNBH_3$ reduction of Schiff bases formed between autoreactive aldehyde groups in beads and free amino groups in the enzyme. Similarly, the enzyme was attached to carboxylate-modified SeraMag beads using one-step carbodiimide chemistry. Kinase activity was minimal in the supernatant obtained after immobilization to BcMag and SeraMag beads (less than 3% of phosphorylated substrate), respectively, suggesting that GSK-3β was completely captured (Fig. 2C+H). On the other hand, 0.2 mg BcMag and SeraMag beads collected immediately after GSK-3β immobilization fully phosphorylated CREB (Fig. 2B) and ε-eIF2B peptides, respectively (Table 1). However, enzyme-loaded BcMag beads lost activity after storage for six days at 4 °C (Fig. 2D), while enzyme-loaded SeraMag beads remained fully active even after 42 days at 4 °C (Fig. 3). There was no statistically significant change of activity of GSK-3β-loaded magnetic SeraMag beads during the storage as determined by one-way ANOVA (p = 0.8021).

The excellent reusability of GSK-3β covalently bound to carboxylate-modified SeraMag beads led us to test the same immobilization strategy for ERK2. The activity of soluble and SeraMag-immobilized tag-free ERK2 was confirmed by phosphorylation of the TH 24-33 peptide in Reaction Buffers I (data not shown) and II (Fig. 4, Table 1).

Operational stability of kinase-loaded SeraMag beads

The operational stability of kinases immobilized to carboxylated SeraMag beads was tested twice a day, 12 hours apart, over five days. After 10 assays, the activity of immobilized GSK-3β was essentially intact at 95.5 ± 0.34% of soluble activity, as calculated from fractions of singly phosphorylated ε-eIF2B peptide with *m*/*z* 1991.98, and of the doubly

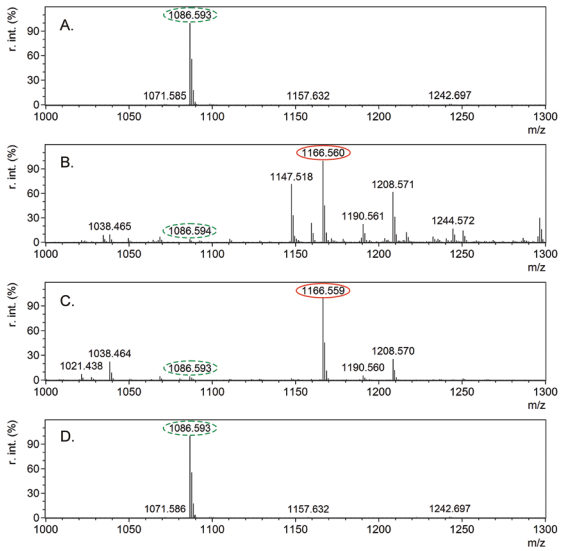


Fig. 4 Representative MALDI-LTQ Orbitrap mass spectra of (A) non-phosphorylated TH 24-33, an ERK2-specific low-molecular weight substrate, and TH 24-33 phosphorylated, for 6 h at 30 °C in Reaction Buffer II supplemented with (B) soluble ERK2, (C) 0.2 mg ERK2-loaded SeraMag beads, and (D) supernatant obtained after ERK2 immobilization onto SeraMag beads. Non-phosphorylated peptides are marked with a green dashed circle, and phosphorylated forms are circled in red circle. Spectra A–C were obtained on MALDI-LTQ Orbitrap XL.

phosphorylated peptide with m/z 2071.94 (Fig. 5A). We observe a slight increase of enzyme activity with the number of cycles performed, namely the phosphorylate rate (%) varies in the range of approximately 4% (Fig. 5A). This phenomenon can be explained by activation and stabilization of immobilized form of GSK-3β by its substrate, which we also observed with other enzymes previously.[44] In contrast, ERK2-loaded SeraMag beads were less operationally stable, with activity decreasing to 36.2 ± 2.01% in the tenth assay, as calculated from the fractions of non-phosphorylated TH 24-33 peptide with m/z 1086.59, and of the phosphorylated peptide with m/z 1166.56 (Fig. 5B). There was a statistically significant change of activity of both kinase-loaded magnetic SeraMag beads when they were used repeatedly in cycles (operational stability) as determined by one-way ANOVA ($p < 0.001$).

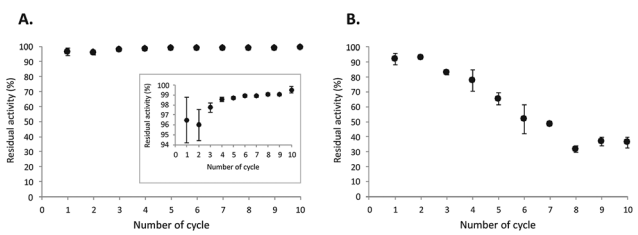


Fig. 5 Operational stability of SeraMag beads loaded with (A) GSK-3β and (B) ERK2. GSK-3β activity was measured in terms of the relative intensities of singly and doubly phosphorylated ε-eIF2B with m/z 1991.98 and 2071.94, respectively. Similarly, ERK2 activity was measured in terms of the relative intensities of unphosphorylated and phosphorylated TH 24-33 with m/z 1086.59 and 1166.56 respectively. The relative intensities of phosphorylated and non-phosphorylated forms were summed and set as 100%, to which the proportion of phosphorylated forms was normalized. Data were collected in triplicate, and are plotted as mean ± SD. There was a statistically significant change of both enzyme activities in time as determined by one-way ANOVA ($p < 0.001$). Inset: Axis *y* zoomed.

Phosphorylation of tau by immobilized kinases

While SeraMag beads with covalently attached kinases are clearly reusable against low-molecular weight substrates, mass transfer effects may have nevertheless affected the quality of immobilized enzymes. Thus, enzyme-loaded beads were tested against full-length microtubule-associated tau, a natively unfolded protein, the phosphorylation of which has been intensively characterized as the main post-translational modification implicated in physiological and pathological processes.[26] Of note, altered GSK-3β activity is associated with abnormal hyperphosphorylation of tau, highlighting its suitability as a model substrate.[45] In addition, GSK-3β has enhanced affinity for pre-phosphorylated substrates with motif SXXXpS, where S is serine, X is any residue, and pS is phosphoserine.[24,25] Accordingly, pre-phosphorylation by ERK2, for instance, may influence GSK-3β activity against Ser/Thr residues in tau.[26]

Sequential phosphorylation by several soluble kinases often requires denaturation steps between cycles to quench the activity of the previous enzyme. However, such steps may also alter the properties and structure not only of the enzyme, but also of the substrate.[46,47] For example, heat denaturation of ERK2 prior to addition of GSK-3β (ESI, Method S3†) resulted in slight differences in the mobility and band intensity of the final product on western blots (Fig. 6A–D, lanes 2–3), in com-

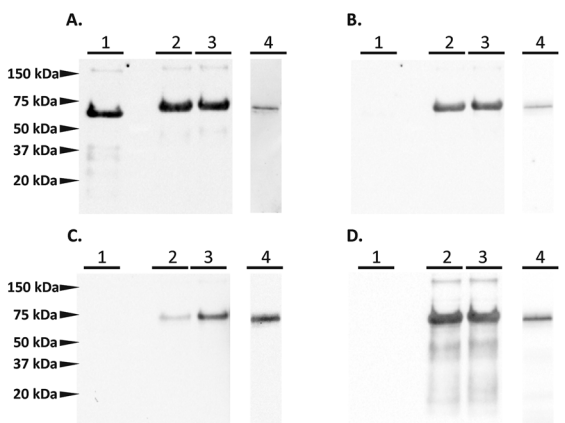


Fig. 6 Detection of phosphorylated tau 1-441 by western blot using (A) monoclonal mouse antibody to tau (tau 46.1, 1 : 60 000), (B) monoclonal mouse antibody to tau phosphorylated at Thr231 (AT180, 1 : 2000), (C) polyclonal rabbit antibody to tau phosphorylated at Ser356 (S356, 1 : 2000), and (D) polyclonal rabbit antibody to tau phosphorylated at Ser396 (S396, 1 : 2000). Lane 1, tau 1-441; 2–3, tau 1-441 phosphorylated by soluble ERK2 and soluble GSK-3β with (2) or without (3) an intermediate denaturing step at 95 °C for 10 min; 4, tau 1-441 phosphorylated by ERK2-loaded SeraMag particles, and then by SeraMag beads loaded with GSK-3β.

This journal is © The Royal Society of Chemistry 2018

parison to products obtained without an intermediate denaturation step.

In this case, separation of phosphorylated products from kinase-loaded magnetic particles would be an easier and helpful alternative. Thus, tau was phosphorylated sequentially with SeraMag beads loaded with ERK2 and GSK-3β. Phosphorylation of specific positions T231 (Fig. 6B, lane 4), S356 (Fig. 6C, lane 4), and S396 (Fig. 6D, lane 4) in tau protein was confirmed by western blot using antibodies to tau and phosphorylated tau characterized in Experimental section (Fig. 6). In addition, tryptic digestion, TiO_2 enrichment, and microgradient separation of the final product generated 26 phosphopeptides on mass spectrometry, 20 of which contained two or more phosphorylations (data not shown). Mapping of phosphorylation sites is ongoing.

Conclusions

We have loaded superparamagnetic beads with functionally active and stable enzymes that phosphorylate target peptides and proteins *in vitro*. In particular, GSK-3β and ERK2 covalently immobilized to carboxylated SeraMag beads have suitable operational stability as reusable kinases against low molecular-weight substrates. Importantly, the magnetic beads enable sequential phosphorylation of proteins without interference among kinases, thereby preserving enzyme activity and specificity. We anticipate that our approach can be adapted for other protein substrates where the reaction with MAP kinases is also required to pre-phosphorylate substrates for efficient, subsequent phosphorylation by GSK-3.[48,49] In addition, kinases immobilized to magnetic beads are easily removed to stop reactions or to minimize contamination of phosphorylated products, and are low-cost due to reusability. Thus, enzyme-loaded magnetic beads may prove effective as a tool to modify various recombinant proteins that require phosphorylation for folding, stability, and biological activity. In some cases, proteins with well-defined phosphorylation at multiple sites may facilitate studies of specific pathological processes.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the research project GACR P304/12/G069, TACR GAMA TG02010058 with the part 01/021 – TG361021, and by Charles University grant Progres Q26.

Notes and references

1 A. L. Nel, N. Minc, C. Smadja, M. Slovakova, Z. Bilkova, J.-M. Peyrin, J.-L. Viovy and M. Taverna, *Lab Chip*, 2008, **8**, 294–301.
2 V. Zlateski, R. Fuhrer, F. M. Koehler, S. Wharry, M. Zeltner, W. J. Stark, T. S. Moody and R. N. Grass, *Bioconjugate Chem.*, 2014, **25**, 677–684.
3 C. Fan, Z. Shi, Y. Pan, Z. Song, W. Zhang, X. Zhao, F. Tian, B. Peng, W. Qin, Y. Cai and X. Qian, *Anal. Chem.*, 2014, **86**, 1452–1458.
4 A. Mukhopadhyay and K. Chakrabarti, *RSC Adv.*, 2015, **5**, 89346–89362.
5 V. E. Bosio, G. A. Islan, Y. N. Martínez, N. Durán and G. R. Castro, *Crit. Rev. Biotechnol.*, 2016, **36**, 447–464.
6 J. Krenkova, A. Szekrenyes, Z. Keresztessy, F. Foret and A. Guttman, *J. Chromatogr. A*, 2013, **1322**, 54–61.
7 A. Paulus, C. Morhardt, N. Lehle and M. Franzreb, *J. Mol. Catal. B: Enzym.*, 2014, **110**, 165–170.
8 T. L. Ogorzalek, S. Wei, Y. Liu, Q. Wang, C. L. Brooks, Z. Chen and E. N. G. Marsh, *Langmuir*, 2015, **31**, 6145–6153.
9 C. B. Park and D. S. Clark, *Biotechnol. Bioeng.*, 2002, **78**, 229–235.
10 Y. Li, J. Xu, Y. Chen, Z. Mei and Y. Xiao, *J. Chromatogr. A*, 2015, **1425**, 8–16.
11 H. Yoshida, C. J. Hastie, H. McLauchlan, P. Cohen and M. Goedert, *J. Neurochem.*, 2004, **90**, 352–358.
12 H. Cao and R. Lin, *Protein J.*, 2008, **27**, 163–169.
13 L. Korecka, B. Jankovicova, J. Krenkova, L. Hernychova, M. Slovakova, A. Le-Nell, J. Chmelik, F. Foret, J.-L. Viovy and Z. Bilkova, *J. Sep. Sci.*, 2008, **31**, 507–515.
14 M. Slovakova, M. Sedlak, B. Krizkova, R. Kupcik, R. Bulanek, L. Korecka, C. Drasar and Z. Bilkova, *Process Biochem.*, 2015, **50**, 2088–2098.
15 T. Hunter, *Cell*, 2000, **100**, 113–127.
16 M. Cargnello and P. P. Roux, *Microbiol. Mol. Biol. Rev.*, 2011, **75**, 50–83.
17 A. Cavallini, S. Brewerton, A. Bell, S. Sargent, S. Glover, C. Hardy, R. Moore, J. Calley, D. Ramachandran, M. Poidinger, E. Karran, P. Davies, M. Hutton, P. Szekeres and S. Bose, *J. Biol. Chem.*, 2013, **288**, 23331–23347.
18 M. Raman, W. Chen and M. H. Cobb, *Oncogene*, 2007, **26**, 3100–3112.
19 C. A. Grimes and R. S. Jope, *Prog. Neurobiol.*, 2001, **65**, 391–426.
20 T. G. Boulton and M. H. Cobb, *Cell Regul.*, 1991, **2**, 357–371.
21 J. M. Boggs, G. Rangaraj, W. Gao and Y.-M. Heng, *Biochemistry*, 2006, **45**, 391–401.
22 H. Qi, S. Prabakaran, F.-X. Cantrelle, B. Chambraud, J. Gunawardena, G. Lippens and I. Landrieu, *J. Biol. Chem.*, 2016, **291**, 7742–7753.
23 J. R. Woodgett, *EMBO J.*, 1990, **9**, 2431–2438.
24 I. Buch, D. Fishelovitch, N. London, B. Raveh, H. J. Wolfson and R. Nussinov, *Biochemistry*, 2010, **49**, 10890–10901.
25 R. Dajani, E. Fraser, S. M. Roe, N. Young, V. Good, T. C. Dale and L. H. Pearl, *Cell*, 2001, **105**, 721–732.
26 L. Martin, X. Latypova, C. M. Wilson, A. Magnaudeix, M.-L. Perrin, C. Yardin and F. Terro, *Ageing Res. Rev.*, 2013, **12**, 289–309.

27 F. Zhang, C. J. Phiel, L. Spece, N. Gurvich and P. S. Klein, *J. Biol. Chem.*, 2003, **278**, 33067–33077.
28 M. Goedert, R. Jakes, R. A. Crowther, P. Cohen, E. Vanmechelen, M. Vandermeeren and P. Cras, *Biochem. J.*, 1994, **301**(Pt 3), 871–877.
29 P. Aqai, N. G. Blesa, H. Major, M. Pedotti, L. Varani, V. E. V. Ferrero, W. Haasnoot and M. W. F. Nielen, *Anal. Bioanal. Chem.*, 2013, **405**, 9427–9436.
30 J. E. Hale, *Anal. Biochem.*, 1995, **231**, 46–49.
31 E. Zatloukalová and Z. Kucerová, *J. Sep. Sci.*, 2006, **29**, 1082–1087.
32 L. Peng, G. J. Calton and J. W. Burnett, *Appl. Biochem. Biotechnol.*, 1987, **14**, 91–99.
33 B. Jankovicova, S. Rosnerova, M. Slovakova, Z. Zverinova, M. Hubalek, L. Hernychova, P. Rehulka, J.-L. Viovy and Z. Bilkova, *J. Chromatogr. A*, 2008, **1206**, 64–71.
34 J. W. Haycock, N. G. Ahn, M. H. Cobb and E. G. Krebs, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 2365–2369.
35 L. Hromadkova, M. Kolarova, B. Jankovicova, A. Bartos, J. Ricny, Z. Bilkova and D. Ripova, *J. Neuroimmunol.*, 2015, **289**, 121–129.
36 J. Gobom, E. Nordhoff, E. Mirgorodskaya, R. Ekman and P. Roepstorff, *J. Mass Spectrom.*, 1999, **34**, 105–116.
37 L. Hromadkova, R. Kupcik, B. Jankovicova, T. Rousar, D. Ripova and Z. Bilkova, *J. Sep. Sci.*, 2016, **39**, 799–807.
38 E. Bowley, E. Mulvihill, J. C. Howard, B. J. Pak, B. S. Gan and D. B. O'Gorman, *BMC Biochem.*, 2005, **6**, 29.
39 *R Core team: a language and environment for statistical computing. Version 3.1. 3*, R Foundation for Statistical Computing, Vienna, Austria, 2015, 2013. Available from: http://R-project.org/.
40 W. Tischer and F. Wedekind, in *Biocatalysis - From Discovery to Application*, ed. P. D. W.-D. Fessner, A. Archelas, D. C. Demirjian, R. Furstoss, H. Griengl, K.-E. Jaeger, E. Morís-Varas, R. Öhrlein, M. T. Reetz, J.-L. Reymond, M. Schmidt, S. Servi, P. C. Shah, W. Tischer and F. Wedekind, Springer, Berlin Heidelberg, 1999, pp. 95–126.
41 C. You and J. Piehler, *Anal. Bioanal. Chem.*, 2014, **406**, 3345–3357.
42 N. Sohrabi, N. Rasouli and M. Torkzadeh, *Chem. Eng. J.*, 2014, **240**, 426–433.
43 S. Ding, A. A. Cargill, I. L. Medintz and J. C. Claussen, *Curr. Opin. Biotechnol.*, 2015, **34**, 242–250.
44 L. Korecka, J. Jezova, Z. Bilkova, M. Benes, D. Horak, O. Hradcova, M. Slovakova and J.-L. Viovy, *J. Magn. Magn. Mater.*, 2005, **293**, 349–357.
45 M. Maqbool, M. Mobashir and N. Hoda, *Eur. J. Med. Chem.*, 2016, **107**, 63–81.
46 P. J. Davis and S. C. Williams, *Allergy*, 1998, **53**, 102–105.
47 K. Murayama and M. Tomida, *Biochemistry*, 2004, **43**, 11526–11532.
48 L. S. Coles, L. Lambrusco, J. Burrows, J. Hunter, P. Diamond, A. G. Bert, M. A. Vadas and G. J. Goodall, *FEBS Lett.*, 2005, **579**, 5372–5378.
49 H. Demagny, T. Araki and E. M. De Robertis, *Cell Rep.*, 2014, **9**, 688–700.

 This journal is © The Royal Society of Chemistry 2018

DISCUSSION

Tau protein is intensively studied protein as its abnormally modified and misfolded forms are the main component of paired helical filaments (Wood et al., 1986; Delacourte and Defossez, 1986). These aggregated filamentous structures form intracellular neurofibrillary tangles, beside Aβ-composed senile plaques, the most relevant histopathological hallmark of Alzheimer´s disease (AD) (Ittner and Gotz, 2011). To date, no cure for AD is available so far. Since the immune system involvement in AD is the strongly discussed topic, antibody-based immunotherapy is under investigation as one of the promising approaches in AD therapy. Especially, natural antibodies reactive with both Aβ peptides and tau protein are in the forefront of interest (Wisniewski and Goni, 2015). Their potential functions in AD progression have to be better established and the clarification whether their occurrence is beneficial or rather harmful is still missing.

The main aim of this thesis was a characterization of natural antibodies against tau protein occurring in IVIG preparation Flebogamma and in pooled plasma samples of AD diagnosed patients as well as of age-matched healthy controls. Related to this topic, properties of tau as a natively unfolded protein and the behavior of some specific tau fragments after *in vitro* digestion were also investigated.

The presence of naturally occurring tau-reactive antibodies (nTau-Abs) in sera of cognitively normal individuals as well as of AD patients was proven in several publications (Rosenmann et al., 2006; Fialova et al., 2011; Bartos et al., 2012; Klaver et al., 2017; Krestova et al., 2017a; Kuhn et al., 2018). Moreover, antibodies directed to tau protein were also detected in intravenous immunoglobulin (IVIG) products (Smith et al., 2013; Smith et al., 2014; Loeffler et al., 2015; Hromadkova et al., 2015; Krestova et al., 2017a) which are widely used in treatment of variable range of diseases, from impaired autoimmunity to neurological disorders (Durandy et al., 2009; Zivkovic, 2016). Especially, since AD was proposed to be a protein conformational disorder, antibody-based immunotherapy directed against altered structurally modified and aggregated proteins become a spotlight in ongoing therapeutic approaches (Wisniewski and Goni, 2015). IVIG products have been tested in several clinical trials with AD and MCI patients, some

of these studies are still ongoing (Boada et al., 2016; Cummings et al., 2017). The pathological changes occurring during MCI and AD progression are more complex and not strictly tied to one particular molecular mechanism as well as IVIG products contain immunoglobulins from thousands of healthy donors which represent the reservoir of natural plasma antibodies directed to many antigens, including numerous self-antigens. Even so, completed trials with IVIG for AD treatment did not provide satisfactory results (Relkin et al., 2014; Relkin et al., 2017). This has triggered the desire for a deeper knowledge of naturally occurring antibodies presented in IVIGs that are specifically directed to AD pathologically relevant proteins. This understanding may also help to contribute to the concept of the production of so-called AD-specific IVIG preparations suggested as an alternative approach for AD treatment (Loeffler, 2014). When we consider structurally modified toxic forms of Aβ peptides and tau protein are the main molecules targeted by AD therapies, it is crucial to evaluate whether natural antibodies directed to these proteins may have a beneficial effect. Until recently, more work in this field was dedicated to natural Aβ peptide-reactive antibodies which were isolated and their mechanism of action tested in cell-based systems as well as in animal models (reviewed in book chapter Bach and Dodel, 2012). On the other hand, tau pathology is partly an independent process from Aβ biology alterations and better correlates with AD progression (Peterson and Sigurdsson, 2015). Thus tau protein is at a forefront of interest as a therapeutic target. Both monoclonal and polyclonal anti-tau antibodies have been produced and intensively tested for their putative protective mechanism whether they may halt tau-associated AD pathology via participating in clearance of altered misfolded tau forms, degradation of oligomeric and aggregated tau species, inhibition of tau polymerization and prevention of neurotoxicity (Schroeder et al., 2016).

To our best knowledge, we provide the first insight into the character and reactive profile of nTau-Abs directly isolated from IVIG product (Hromadkova et al., 2015; Krestova et al., 2017a, b). Flebogamma DIF (Grifols Biologicals Inc., Los Angeles, CA, USA), IVIG preparation (Flebogamma DIF 5%, Grifols Biologicals Inc., Los Angelos, USA) examined in ongoing phase III trial (https://clinicaltrials.gov/ct2/show/NCT01561053), was selected for isolation of nTau-Abs by affinity chromatography with human full-length tau (1-441 aa) as a ligand (Krestova et al., 2017b). The comparison of IgG subclasses distribution in original IVIG sample and isolated fraction indicates skewing toward IgG3 in IgG fraction enriched in nTau-Abs. These results may be interesting due to the fact that IgG3 subclass generally belongs to potent pro-inflammatory

antibodies in early response to an antigen with a short half-life and natural Abs are mostly IgG3 subclass-specific (Panda and Ding, 2015). Since NFT formation is known as a dynamic process in which tau protein is abundantly post-translationally modified, the reactivity of isolated nTau-Abs was examined against several recombinant tau forms. Truncation and phosphorylation have been considered as the main tau PTMs enhancing the development of tau pathology in AD. Reasonably, recombinant human full-length tau proteins differing by the presence of 6x histidine-tag and two truncated forms corresponding to tau amino acid sequences 13-391 and 155-421 in both non-phosphorylated/phosphorylated states, respectively, were applied. Interestingly, the non-phosphorylated tau fragment (155-421 aa) demonstrated the highest reactivity and even avidity index with isolated nTau-Abs. The pro-aggregation truncation at Asp421 mediated by caspases, mostly by caspase-3, always precedes Glu391 cleavage event and is linked to cell toxicity (Nicholls et al., 2017). Much less is known about the N-terminal truncation occurring in tau molecule but thrombin seems to be an endogenous protease candidate of tau cleavage at Arg155 as was proven *in vitro* (Olesen, 1994). This fragment had high reactivity with antibodies in all tested antibody fractions (original IVIG sample, flow-through fraction and elution fraction enriched with nTau-Abs) thus we can assume that this truncated tau form containing proline-rich regions and microtubule-binding domain with all four repeat domains prone to aggregate (Okuyama et al., 2008; Sugino et al., 2009) could be highly antigenic also in healthy population.

Based on the latest studies which revealed that oligomeric forms of altered tau molecules may be the most toxic species significantly contributing to spreading of tau pathology (Guerrero-Munoz et al., 2015), the investigation of the characteristics and reactivity of plasma nTau-Abs against native physiological and pathological tau forms is needed. We extended our previous study with nTau-Abs to investigate their reactivity with native tau proteins present in brain homogenates of controls and histopathologically proven AD patients (Krestova et al., 2017a). We isolated a new batch of nTau-Abs from IVIG product Flebogamma (nTau-IVIG Abs), and furthermore from pooled plasma samples obtained from patients with diagnosed AD (nTau-AD Abs) and age-matched cognitively normal individuals, referred to healthy controls (nTau-Ctrl Abs). Results with natural Abs isolated from IVIG and controls confirmed the previous findings with tau fragment (155-421 aa) in its non-phosphorylated form showing stronger reactivity. In contrary, nTau-AD Abs has higher reactivity with recombinant human full-length tau.

To our surprise, the avidity index was approximately the same for all three newly isolated nTau-Abs fraction (nTau-IVIG, nTau-AD, and nTau-Ctrl Abs) and previously isolated nTau-Abs from IVIG with this tau fragment 155-421. More interestingly, we observed differences in reactivity of isolated nTau-Abs with brain homogenates. The nTau-AD Abs reacted mostly to monomeric tau forms in brain homogenates in comparison to nTau-IVIG and nTau-Ctrl Abs, which preferably bound to more aggregated tau forms. These results support the concept that immune system may be involved in controlling of occurrence of pathological protein forms under physiological conditions. To date, the physiological release of endogenous tau protein into the peripheral blood system mediated by neuronal activity and with the participation of glymphatic pathway was described (Iliff et al., 2014; Benveniste et al., 2017). Tau protein occurring in a periphery is likely to be subjected to fast degradation and elimination by natural antibodies and this potential mechanism may be altered during the development of AD. But more studies are needed to prove the hypothesis suggesting impaired repertoire of naturally occurring antibodies against misfolded and oligomeric/aggregated forms of proteins playing a crucial role, which are gained to toxic function in AD pathology.

In connection with the project of natural tau-reactive antibodies, we optimized epitope mapping methods to determine the precise localization of interaction sides between tau protein and selected tau-reactive antibodies. In our published original paper, we applied two magnetic-bead-based epitope mapping approaches as a part of the quality evaluation of monoclonal anti-tau antibodies for immunomagnetic purification of native tau protein (Jankovicova et al., 2014; Jankovicova et al., 2015). These methodological approaches were epitope extraction and epitope excision, both based on bioaffinity separation techniques coupled with final mass spectrometry (MS) analysis. In epitope extraction protocol suitable for determination of linear epitopes, the target protein is digested by immobilized proteolytic enzyme(s) and generated fragments are subsequently incubated with specific antibodies bound to the solid phase. Whereas in epitope excision approach used in the identification of both linear and conformational epitopes the immunocomplex formation between entire protein molecules and immobilized antibodies occurs first, followed by protein fragmentation. After washing steps, the epitope-containing fragments interacting with the antibody binding sites are released by acidic elution in both protocols and identified by MS (Jeyarajah et al., 1998; Jankovicova et al., 2008; Paraschiv et al., 2013). Interestingly, despite applied immunosorbent specificity, a tryptic fragment corresponding to tau

amino acid sequence 299-HVPGGGSVQIVYKPVDLSK-317 (theoretical m/z 1980.0912) also occurred in all elution fractions (Jankovicova et al., 2014; Hromadkova et al., 2016). Its peak with high relative intensity complicated the interpretation of MS spectra and was detected even in epitope extraction and excision procedure carried out with negative controls (magnetic beads without bound antibody molecules). Thus, we assumed its presence in elution fractions may be caused by a non-specific sorption linked to its adhesive character. Moreover, these adhesive properties are likely to be responsible for aggregation of tau pathological forms into PHFs. Thus, this study also partly reveal how this behavior of protein observed *in vitro* studies could be associated with events occurring during protein misfolding and aggregation *in vivo*.

Based on the above considerations, if we would like to apply magnetic-bead-based epitope mapping to the determination of tau-reactive antibodies with unknown epitope reactivity as in case of isolated nTau-Abs, the first step has to be avoiding of false positive and negative results from MS analysis (Hromadkova et al., 2016). To preclude the non-specific sorption of this adhesive fragment, we tested several conventional alterations to suppress or significantly reduce the non-specific sorption of this fragment to magnetic particles (MPs) and plastic tubes; modification of MPs surface by BSA or by 30kDa polyethyleneglycol and reductive amination of remaining reactive groups on MPs by Tris or ethanolamine (Hermanson, 2013; Kucerova et al., 2014). The fact that self-aggregation propensity can contribute to non-specific adsorption, we also applied an additional 8M urea incubation step in epitope extraction protocol to disturb the protein-protein interactions participating in aggregate formation.

None of the aforementioned approaches brought the desired effect to eliminate the non-specific sorption and the adhesive fragment 299-HVPGGGSVQIVYKPVDLSK-317 was still present in MS spectra as a high intensive peak. Thus, we focused more on the character of this peptide because it contains the most hydrophobic residue stretch 306-VQIVYKPVDLSKV-318 (Mukrasch et al., 2009) and the most studied pro-aggregation motif PHF6, 306-VQIVYK-311, of tau protein (von Berger et al., 2000, Li and Lee, 2006). We confirmed the hydrophobic character and pro-aggregation propensity of the adhesive fragment by a microscale RP-LC experiment (Fekete et al., 2015) and Thioflavin S assay (Chirita et al., 2015). We supposed that both these characteristics can be enhanced after tryptic digestion and contribute to adsorption on MPs surface (Hromadkova et al., 2016).

To support our hypothesis, we suggested a proteolytic system which can abolish both the pro-aggregation motif as well as the concentrated hydrophobic amino acids formation. The combination of immobilized trypsin and α-chymotrypsin tau cleavage was introduced since trypsin still provides proper fragments´ length to cover the tau sequence and α-chymotrypsin effectively cleaves the peptide bound 310-YK-311 within the critical pro-aggregation motif 306-VQIVYK-311. The formation of newly cleaved fragments reduces the adsorption, and the interpretation of MS data is more valid for evaluation of anti-tau antibody epitope specificity. In this already cited paper (Hromadkova et al. 2016), we provided information about the adhesive character of the tryptic tau fragment 299-HVPGGGSVQIVYKPVDLSK-317, containing PHF6 minimal aggregation motif and the most hydrophobic tau region, which was also seen in other papers publishing data with tryptically digested tau (Becker et al., 2007; Becker and Przybylski, 2007).

It seems to be necessary to take into consideration tau-specific properties, which can be enhanced after protein fragmentation and then be associated with significant risk to complicate the structural analysis of tau protein and to give results leading to the erroneous interpretation. These findings are considered in an ongoing project focusing on characterization of natural tau-reactive antibodies where a part is dedicated to epitope mapping to reveal some potential differences in their reactivity depending on their source, particularly isolated from IVIG preparation, sera of AD and age-matched cognitively normal controls.

In both studies regarding isolated naturally occurring antibodies directed to tau protein, it was surprising that phosphorylation partly abolished their reactivity with all recombinant tau forms applied (Hromadkova et al., 2015; Krestova et al., 2017a) despite the fact that abnormally phosphorylated tau protein is a main component of NFTs in AD brains (Grundke-Iqbal et al., 1986). Tau protein belongs to the group of microtubule-associated proteins maintaining numerous physiological functions in neurons. Its natively unfolded character possessing only a few sequences with a tendency to take secondary structures gives tau unique properties in sense of variable folding/unfolding states and binding dynamics depending on a huge range of posttranslational modifications (PTMs) (Bah and Forman-Kay, 2016). Phosphorylation is the most significant PTM of tau protein since full-length human tau (2N4R isoform, 1-441 aa) has

85 putative phosphorylation sites. As many studies reported, the site-specific phosphorylation of tau is closely related to the balance between tau physiological and pathological functions. Tau is phosphorylated under both physiological and AD-associated pathological conditions but the phosphorylation rate and specific phosphorylated residues significantly differ (Kopke et al.; 1993, Martin et al., 2013; Noble et al., 2013). Moreover, truncation of tau phosphorylated only in a few specific sites rather than hyperphosphorylated form is considered to be a very early event in tangle formation (Mondragon-Rodriguez et al., 2008). Moreover, tau phosphorylation at certain residues may have a protective effect and prevent the assembly of tau into filamentous structures (Schneider et al., 1999). To date, there is no commercially available recombinant tau protein phosphorylated in a defined manner that could be applicable in biochemical and immunoanalytical sensitive assays. Also, it is necessary to admit the main limitation of our studies - the use of recombinant human full-length tau protein as a ligand in antibody isolation procedure. Thus, the amount of nTau-Abs directed to more specific phosphorylated forms may be affected and more native-like tau protein is needed for the isolation process itself in future studies.

All these above-mentioned reasons led us to figure out a task how to obtain tau protein with well-defined phosphorylated sites. The first goal, which is also included as a part of this thesis, was to prepare a valid tool for producing of various recombinant full-length/truncated tau proteins with fully defined phosphorylation in a high purity that would serve as ligands in isolation procedures or as antigens in sensitive immunoanalytical assays. We have developed kinase-loaded magnetic particles (MPs) applicable for *in vitro* phosphorylation of tau protein, particularly ERK2 and GSK-3β-loaded magnetic beads (Hromadkova et al., 2018). To our knowledge, the preparation of a system with active forms of both kinases covalently bound onto MPs with great operational and storage stability has not been reported previously. The list of benefits of using such a biotechnological tool comprises the ease of sample handling when kinases immobilized onto magnetic beads can be gently removed, stability and reusability connected with economics and acquisition of phosphorylated products with minimal enzyme contamination compared to those using soluble kinase forms (Li et al., 2015). Also, hierarchical phosphorylation of tau with specific phosphorylation sites priming for further phosphorylation in nearby residues and various kinase interactions, which influence their specificity (Goedert et al., 1994; Hanger et al., 2007), can be better control by utilizing kinase-loaded MPs.

We selected two proline-directed kinases GSK-3β and ERK2 which are involved in tau phosphorylation under both physiological and pathological circumstances (Hanger and Noble, 2011, Drewes et al., 1992). Moreover, tau pre-phosphorylated by ERK2 is a more favorable substrate for subsequent phosphorylation by GSK-3β (Goedert et al., 1994; Zhang et al., 2003), which prefers pre-phosphorylated substrates with motif S/T-XXXpS (Fiol et al., 1987). First of all, we evaluated the maintenance of activity of our kinase-loaded MPs and their stability by using specific low-molecular-weight substrates. By western blot and mass spectrometry analysis connected with TiO_2 enrichment of phosphopeptides and microgradient separation, we proved the successful phosphorylation of recombinant tau protein where 26 phosphopeptides were identified (Hromadkova et al., 2018). The precise mapping of phosphorylated sites under various reaction conditions is ongoing.

The thesis summarizes some promising results of the project which is still ongoing. Future plans based on the research with isolated natural tau-reactive antibodies will address their epitope mapping and study of their mechanisms of actions *in situ* in cell-based systems modeling neurodegeneration. Also, the subclass distribution of these antibodies will be more investigated in relation to their function. In the subproject regarding tau phosphorylation by kinase-loaded magnetic beads, using of magnetic beads decorated with other different kinds of active kinases and their characterization have been investigated (for instance, manuscript regarding PKA-loaded MPs is in preparation). Precise localization of phosphorylated amino acid residues in tau protein by novel nanoscale LC-MS approaches in phosphoproteomics and the effect of various reaction conditions to obtain well-defined phosphorylated tau antigens for further applications are the main questions.

CONCLUSION

The main theme of this thesis is tau protein, one of the most relevant biomarkers of AD.

The thesis is mainly focused on basic characterization of naturally occurring tau-reactive antibodies (nTau-Abs) isolated from plasma samples. We successfully isolated nTau-Abs from pooled plasma obtained from AD patients, age-matched cognitively normal individuals, and from IVIG product Flebogamma. Among these three groups, the basic characterization showed differences in reactivity with applied tau forms varied in their most pathologically relevant post-translational modifications; truncation, phosphorylation, and aggregation, respectively. Our results partly support the concept that the repertoire of nTau-Abs is a natural part of the humoral immune system. This project is ongoing to confirm our hypothesis that the function of nTau-Abs is rather protective via their participation in clearance of putative toxic tau forms and this system is altered in AD as may indicate these results so far. Thus, introduction and optimization of epitope mapping approaches which may unravel the main antigenic structure of modified and misfolded tau are also included. It is necessary to bear in mind that tau protein is natively unfolded protein possessing several regions with the pro-aggregation tendency. We showed that pro-aggregation and hydrophobic properties of tau regions may be enhanced by tau fragmentation which is widely used in proteomic studies and may lead to non-specific sorption of such fragments and erroneous interpretation of results. Thus, we suggested disturbing the key pro-aggregation sequences by a combination of proteases to suppress non-specific sorption of such fragments to obtain more relevant results.

The last sub-project of this thesis was to develop a reusable system for sequential *in vitro* tau phosphorylation to produce highly pure defined product applicable in isolation procedures and sensitive immunoassays with nTau-Abs. For this purpose, we introduced the preparation of kinase-loaded magnetic beads with numerous advantages, such as their reusability with respect to economic aspect, the more controllable process of phosphorylation, and a high purity of final phosphoprotein because of easy removal of active enzyme molecules. This system was successfully applied with tau protein. This part of the project is ongoing and is promisingly developed.

Back to the title “Tau protein, a biomarker of Alzheimer´s disease: *in vitro* phosphorylation and tau-reactive antibodies characterization”, the main aims and goals of the thesis were fulfilled. The discussed results provide novel information about tau protein and natural tau-reactive antibodies in relation to tau posttranslational modifications and Alzheimer´s disease as well as give raise numerous new questions for the further research in this area.

REFERENCES

1. ABRAHA, A., et al. C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. *Journal of Cell Science*, 2000, 113.21: 3737-3745.
2. ALNEMRI, E. S., et al. Human ICE/CED-3 protease nomenclature. *Cell*, 1996, 87.2: 171.
3. ANDO, K., et al. Stabilization of microtubule-unbound tau via tau phosphorylation at Ser262/356 by Par-1/MARK contributes to augmentation of AD-related phosphorylation and Aβ42-induced tau toxicity. *PLoS Genetics*, 2016, 12.3: e1005917.
4. ARRASATE, M.; PEREZ, M.; AVILA, J. Tau dephosphorylation at tau-1 site correlates with its association to cell membrane. *Neurochemical Research*, 2000, 25.1: 43-50.
5. ARAI, T.; GUO, J.-P.; MCGEER, P. L. Proteolysis of non-phosphorylated and phosphorylated tau by thrombin. *Journal of Biological Chemistry*, 2005, 280.7: 5145-5153.
6. ARAI, T., et al. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. *Journal of Neuropathology & Experimental Neurology*, 2006, 65.1: 19-25.
7. ARMSTRONG, R. A. What causes Alzheimer's disease?. *Folia Neuropathologica*, 2013, 51.3: 169-188.
8. ARONOV, S., et al. Axonal tau mRNA localization coincides with tau protein in living neuronal cells and depends on axonal targeting signal. *Journal of Neuroscience*, 2001, 21.17: 6577-6587.
9. ARRIAGADA, P. V., et al. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*, 1992, 42.3: 631-631.
10. ATASSI, M. Z., et al. Molecular mechanisms of autoimmunity. *Autoimmunity*, 2008, 41.2: 123-132.
11. ATZORI, C., et al. Activation of the JNK/p38 pathway occurs in diseases characterized by tau protein pathology and is related to tau phosphorylation but not to apoptosis. *Journal of Neuropathology & Experimental Neurology*, 2001, 60.12: 1190-1197.
12. AUGUSTINACK, J. C., et al. Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. *Acta Neuropathologica*, 2002, 103.1: 26-35.
13. AVRAMEAS, S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunology Today*, 1991, 12.5: 154-159.
14. BACH, J.-P.; DODEL, R. Naturally occurring autoantibodies against β-Amyloid. In: *Naturally Occurring Antibodies (NAbs)*. Springer, New York, NY, 2012. p. 91-99.
15. BAH, A.; FORMAN-KAY, J. D. Modulation of intrinsically disordered protein function by post-translational modifications. *Journal of Biological Chemistry*, 2016, 291.13: 6696-6705.
16. BARTOS, A., et al. Patients with Alzheimer disease have elevated intrathecal synthesis of antibodies against tau protein and heavy neurofilament. *Journal of Neuroimmunology*, 2012, 252.1: 100-105.
17. BASURTO-ISLAS, G., et al. Accumulation of aspartic acid421-and glutamic acid391-cleaved tau in neurofibrillary tangles correlates with progression in Alzheimer disease. *Journal of Neuropathology & Experimental Neurology*, 2008, 67.5: 470-483.
18. BASURTO-ISLAS, G., et al. Activation of asparaginyl endopeptidase leads to Tau hyperphosphorylation in Alzheimer disease. *Journal of Biological Chemistry*, 2013, 288.24: 17495-17507.
19. BATEMAN, R. J., et al. Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease. *Alzheimer's Research & Therapy*, 2011, 3.1: 1.

20. BECKER, W., et al. Sequence characteristics, subcellular localization, and substrate specificity of DYRK-related kinases, a novel family of dual specificity protein kinases. *Journal of Biological Chemistry*, 1998, 273.40: 25893-25902.
21. BECKER, J. S., et al. High resolution mass spectrometric brain proteomics by MALDI-FTICR-MS combined with determination of P, S, Cu, Zn and Fe by LA-ICP-MS. *International Journal of Mass Spectrometry*, 2007, 261.1: 68-73.
22. BECKER, J. S.; PRZYBYLSKI, M. Studies of structure and phosphorylation of tau protein using high resolution mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 2007, 22.7: 761-765.
23. BEDNARSKI, E.; LYNCH, G.. Cytosolic proteolysis of τ by cathepsin D in hippocampus following suppression of cathepsins B and L. *Journal of Neurochemistry*, 1996, 67.5: 1846-1855.
24. BEHREND, L., et al. Interaction of casein kinase 1 delta (CK1δ) with post-Golgi structures, microtubules and the spindle apparatus. *European Journal of Cell Biology*, 2000, 79.4: 240-251.
25. BENVENISTE, H.; LEE, H.; VOLKOW, N. D. The glymphatic pathway: waste removal from the CNS via cerebrospinal fluid transport. *The Neuroscientist*, 2017, 23.5: 454-465.
26. BERRY, R. W., et al. Inhibition of tau polymerization by its carboxy-terminal caspase cleavage fragment. *Biochemistry*, 2003, 42.27: 8325-8331.
27. BIBOW, S., et al. The dynamic structure of filamentous tau. *Angewandte Chemie International Edition*, 2011, 50.48: 11520-11524.
28. BIERNAT, J., et al. Phosphorylation of Ser 262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron*, 1993, 11.1: 153-163.
29. BILLINGSLEY, M. L.; KINCAID, R. L. Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. *Biochemical Journal*, 1997, 323.3: 577-591.
30. BINDER, L. I.; FRANKFURTER, Anthony; REBHUN, Lionel I. The distribution of tau in the mammalian central nervous system. *The Journal of Cell Biology*, 1985, 101.4: 1371-1378.
31. BINDER, L. I., et al. Tau, tangles, and Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 2005, 1739.2: 216-223.
32. BOADA, M., et al. Treatment of Alzheimer disease using combination therapy with plasma exchange and haemapheresis with albumin and intravenous immunoglobulin: Rationale and treatment approach of the AMBAR (Alzheimer Management By Albumin Replacement) study. *Neurología (English Edition)*, 2016, 31.7: 473-481.
33. BOULTON, T. G., et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, 1991, 65.4: 663-675.
34. BRAAK, F.; BRAAK, H.; MANDELKOW, E.-M. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathologica*, 1994, 87.6: 554-567.
35. BRADY, R. M.; ZINKOWSKI, R. P.; BINDER, L. I. Presence of tau in isolated nuclei from human brain. *Neurobiology of Aging*, 1995, 16.3: 479-486.
36. BRANDT, R.; LÉGER, J.; LEE, G. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *The Journal of Cell Biology*, 1995, 131.5: 1327-1340.

37. BRION, J.-P., et al. Developmental Changes in τ Phosphorylation: Fetal τ Is Transiently Phosphorylated in a Manner Similar to Paired Helical Filament-τ Characteristic of Alzheimer's Disease. *Journal of Neurochemistry*, 1993, 61.6: 2071-2080.
38. BUEE, L., et al. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders1. *Brain Research Reviews*, 2000, 33.1: 95-130.
39. CANU, N., et al. Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. *Journal of Neuroscience*, 1998, 18.18: 7061-7074.
40. CAO, C., et al. The ARG tyrosine kinase interacts with Siva-1 in the apoptotic response to oxidative stress. *Journal of Biological Chemistry*, 2001, 276.15: 11465-11468.
41. CARMEL, G., et al. The structural basis of monoclonal antibody Alz50's selectivity for Alzheimer's disease pathology. *Journal of Biological Chemistry*, 1996, 271.51: 32789-32795.
42. CATTEPOEL, S., et al. Effect of IVIG Formulation on IgG Binding to Self-and Exo-Antigens In Vitro and In Vivo. *PloS One*, 2016, 11.8: e0161826.
43. CHEN, Q., et al. Tau protein is involved in morphological plasticity in hippocampal neurons in response to BDNF. *Neurochemistry International*, 2012, 60.3: 233-242.
44. CHESSER, A. S.; PRITCHARD, S.e M.; JOHNSON, G. V. W. Tau clearance mechanisms and their possible role in the pathogenesis of Alzheimer disease. *Frontiers in Neurology*, 2013, 4.
45. CHILDS, T. J.; MAK, A. S. MAP kinases from bovine brain: purification and characterization. *Biochemistry and Cell Biology*, 1993, 71.11-12: 544-555.
46. CHIRITA, C. N., et al. Triggers of full-length tau aggregation: a role for partially folded intermediates. *Biochemistry*, 2005, 44.15: 5862-5872.
47. CHO, J.-H.; JOHNSON, G. V. W. Glycogen Synthase Kinase 3β Phosphorylates tau at both primed and unprimed sites differential impact on microtubule binding. *Journal of Biological Chemistry*, 2003, 278.1: 187-193.
48. CHOI, M. C., et al. Human microtubule-associated-protein tau regulates the number of protofilaments in microtubules: a synchrotron x-ray scattering study. *Biophysical Journal*, 2009, 97.2: 519-527.
49. CHOW, K. M.; GUAN, H.; HERSH, L. B. Aminopeptidases do not directly degrade tau protein. *Molecular Neurodegeneration*, 2010, 5.1: 48.
50. CLEVELAND, D. W.; HWO, S.-Y.; KIRSCHNER, M. W. Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *Journal of Molecular Biology*, 1977, 116.2: 227-247. **a**
51. CLEVELAND, D. W.; HWO, S.-Y.; KIRSCHNER, M. W. Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *Journal of Molecular Biology*, 1977, 116.2: 207-225. **b**
52. COOKE, M. P.; PERLMUTTER, R. M. Expression of a novel form of the fyn proto-oncogene in hematopoietic cells. *The New Biologist*, 1989, 1.1: 66-74.
53. CORREAS, I.; DIAZ-NIDO, J.; AVILA, J. Microtubule-associated protein tau is phosphorylated by protein kinase C on its tubulin binding domain. *Journal of Biological Chemistry*, 1992, 267.22: 15721-15728.
54. COTMAN, C. W., et al. The role of caspase cleavage of tau in Alzheimer disease neuropathology. *Journal of Neuropathology & Experimental Neurology*, 2005, 64.2: 104-112.
55. COUCHIE, D., et al. Microtubule-associated proteins and in vitro astrocyte differentiation. *The Journal of Cell Biology*, 1985, 101.6: 2095-2103.
56. COUCHIE, D., et al. Primary structure of high molecular weight tau present in the peripheral nervous system. *Proceedings of the National Academy of Sciences*, 1992, 89.10: 4378-4381.

57. CRUZ, J. C.; TSAI, L.-H.. Cdk5 deregulation in the pathogenesis of Alzheimer's disease. *Trends in Molecular Medicine*, 2004, 10.9: 452-458.
58. CUENDA, A.; ROUSSEAU, S. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2007, 1773.8: 1358-1375.
59. CUMMINGS, J., et al. Alzheimer's disease drug development pipeline: 2017. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, 2017, 3.3: 367-384.
60. DAVID, D. C., et al. Proteasomal degradation of tau protein. *Journal of Neurochemistry*, 2002, 83.1: 176-185.
61. DE CALIGNON, A., et al. Caspase activation precedes and leads to tangles. *Nature*, 2012, 464.7292: 1201.
62. DELACOURTE, A.; DEFOSSEZ, A. Alzheimer's disease: Tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. *Journal of the Neurological Sciences*, 1986, 76.2: 173-186.
63. DERKINDEREN, P., et al. Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase. *Journal of Neuroscience*, 2005, 25.28: 6584-6593.
64. DIERSSEN, M.; DE LAGRÁN, M. M. DYRK1A (dual-specificity tyrosine-phosphorylated and-regulated kinase 1A): a gene with dosage effect during development and neurogenesis. *The Scientific World Journal*, 2006, 6: 1911-1922.
65. DREWES, G., et al. Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. *The EMBO Journal*, 1992, 11.6: 2131.
66. DREWES, G., et al. Dephosphorylation of tau protein and Alzheimer paired helical filaments by calcmeurin and phosphatase-2A. *FEBS Letters*, 1993, 336.3: 425-432.
67. DUNNING, C. J., et al. Direct high affinity interaction between Aβ42 and GSK3α stimulates hyperphosphorylation of Tau. A new molecular link in Alzheimer's disease?. *ACS Chemical Neuroscience*, 2015, 7.2: 161-170.
68. DURANDY, A., et al. Intravenous immunoglobulins–understanding properties and mechanisms. *Clinical & Experimental Immunology*, 2009, 158.s1: 2-13.
69. DING, H.; MATTHEWS, T. A.; JOHNSON, G. V. W. Site-specific phosphorylation and caspase cleavage differentially impact tau-microtubule interactions and tau aggregation. *Journal of Biological Chemistry*, 2006, 281.28: 19107-19114.
70. DIXIT, R., et al. Differential regulation of dynein and kinesin motor proteins by tau. *Science*, 2008, 319.5866: 1086-1089.
71. DRUBIN, D. G.; KIRSCHNER, M. W. Tau protein function in living cells. *The Journal of Cell Biology*, 1986, 103.6: 2739-2746.
72. ELIE, A., et al. Tau co-organizes dynamic microtubule and actin networks. *Scientific Reports*, 2015, 5.
73. ENGMANN, O.; GIESE, K. P. Crosstalk between Cdk5 and GSK3β: implications for Alzheimer's Disease. *Frontiers in Molecular Neuroscience*, 2009, 2. 838.
74. FASULO, L., et al. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. *Journal of Neurochemistry*, 2000, 75.2: 624-633.
75. FEKETE, S., et al. Adsorption and recovery issues of recombinant monoclonal antibodies in reversed-phase liquid chromatography. *Journal of Separation Science*, 2015, 38.1: 1-8.
76. FERREIRA, A.; BIGIO, E. H. Calpain-mediated tau cleavage: a mechanism leading to neurodegeneration shared by multiple tauopathies. *Molecular Medicine*, 2011, 17.7-8: 676.

77. FERRER, I., et al. Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are differentially expressed in tau deposits in neurons and glial cells in tauopathies. *Journal of Neural Transmission*, 2001, 108.12: 1397-1415.
78. FERRER, I., et al. Constitutive Dyrk1A is abnormally expressed in Alzheimer disease, Down syndrome, Pick disease, and related transgenic models. *Neurobiology of Disease*, 2005, 20.2: 392-400.
79. FIALOVA, L., et al. Increased intrathecal high-avidity anti-tau antibodies in patients with multiple sclerosis. *PLoS One*, 2011, 6.11: e27476.
80. FIOL, C. J., et al. Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *Journal of Biological Chemistry*, 1987, 262.29: 14042-14048.
81. FISCHER, A., et al. Opposing roles of transient and prolonged expression of p25 in synaptic plasticity and hippocampus-dependent memory. *Neuron*, 2005, 48.5: 825-838
82. FRAME, S.; COHEN, P. GSK3 takes centre stage more than 20 years after its discovery. *Biochemical Journal*, 2001, 359.1: 1-16.
83. FRANCIS, P. T., et al. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *Journal of Neurology, Neurosurgery & Psychiatry*, 1999, 66.2: 137-147.
84. FROST, B., et al. Tau promotes neurodegeneration through global chromatin relaxation. *Nature Neuroscience*, 2014, 17.3: 357-366.
85. GAMBLIN, T. C., et al. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 2003, 100.17: 10032-10037.
86. GAMBLIN, T. C. Potential structure/function relationships of predicted secondary structural elements of tau. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 2005, 1739.2-3: 140-149.
87. GARCÍA-SIERRA, F., et al. Accumulation of C-terminally truncated tau protein associated with vulnerability of the perforant pathway in early stages of neurofibrillary pathology in Alzheimer's disease. *Journal of Chemical Neuroanatomy*, 2001, 22.1: 65-77.
88. GARCIA-SIERRA, F., et al. Conformational changes and truncation of tau protein during tangle evolution in Alzheimer's disease. *Journal of Alzheimer's Disease*, 2003, 5.2: 65-77.
89. GARG, S., et al. Cleavage of Tau by calpain in Alzheimer's disease: the quest for the toxic 17 kD fragment. *Neurobiology of Aging*, 2011, 32.1: 1-14.
90. GEORGIEFF, I. S., et al. High molecular weight tau: preferential localization in the peripheral nervous system. *Journal of Cell Science*, 1991, 100.1: 55-60.
91. GHANNAD, M. S., et al. Alzheimer's disease and the role of infectious Agents: A review. *Journal of Chemical and Pharmaceutical Sciences*, 2016, 9.1: 46-53.
92. GHOSHAL, N., et al. A new molecular link between the fibrillar and granulovacuolar lesions of Alzheimer's disease. *The American Journal of Pathology*, 1999, 155.4: 1163-1172.
93. GHOSHAL, N., et al. Tau-66: evidence for a novel tau conformation in Alzheimer's disease. *Journal of Neurochemistry*, 2001, 77.5: 1372-1385.
94. GIESE, K. P.; RIS, L.; PLATTNER, F. Is there a role of the cyclin-dependent kinase 5 activator p25 in Alzheimer's disease?. *Neuroreport*, 2005, 16.16: 1725-1730.
95. GIESE, K. P. Generation of the Cdk5 activator p25 is a memory mechanism that is affected in early Alzheimer's disease. *Frontiers in Molecular Neuroscience*, 2014, 7.

96. GOEDERT, M., et al. Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *The EMBO Journal*, 1989, 8.2: 393.
97. GOEDERT, M.; JAKES, R. Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *The EMBO Journal*, 1990, 9.13: 4225.
98. GOEDERT, M.; SPILLANTINI, M. G.; CROWTHER, R. A. Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system. *Proceedings of the National Academy of Sciences*, 1992, 89.5: 1983-1987.
99. GOEDERT, M., et al. Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein. *Biochemical Journal*, 1994, 301.3: 871-877.
100. GONG, C.-X., et al. Phosphoprotein phosphatase activities in Alzheimer disease brain. *Journal of Neurochemistry*, 1993, 61.3: 921-927.
101. GONG, C.-X., et al. Alzheimer's Disease Abnormally Phosphorylated τ Is Dephosphorylated by Protein Phosphatase-2B (Calcineurin). *Journal of Neurochemistry*, 1994, 62.2: 803-806. **a**
102. GONG, C.-X.; GRUNDKE-IQBAL, I.; IQBAL, K. Dephosphorylation of Alzheimer's disease abnormally phosphorylated tau by protein phosphatase-2A. *Neuroscience*, 1994, 61.4: 765-772. **b**
103. GONG, C.-X., et al. Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and-2C and its implication in Alzheimer disease. *FEBS Letters*, 1994, 341.1: 94-98. **c**
104. GONG, C.-X., et al. Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain implications for neurofibrillary degeneration in Alzheimer's disease. *Journal of Biological Chemistry*, 2000, 275.8: 5535-5544.
105. GONG, C.-X., et al. Post-translational modifications of tau protein in Alzheimer's disease. *Journal of Neural Transmission*, 2005, 112.6: 813-838.
106. GONG, C.-X., et al. Dysregulation of protein phosphorylation/dephosphorylation in Alzheimer's disease: a therapeutic target. *Journal of Biomedicine and Biotechnology*, 2006, 1-11.
107. GRUNDKE-IQBAL, I., et al. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences*, 1986, 83.13: 4913-4917.
108. GRUNE, T., et al. Tau protein degradation is catalyzed by the ATP/ubiquitin-independent 20S proteasome under normal cell conditions. *Archives of Biochemistry and Biophysics*, 2010, 500.2: 181-188.
109. GUEDJ, F., et al. DYRK1A: a master regulatory protein controlling brain growth. *Neurobiology of Disease*, 2012, 46.1: 190-203.
110. GUERRERO-MUÑOZ, M. J.; GERSON, J.; CASTILLO-CARRANZA, D. L. Tau oligomers: the toxic player at synapses in Alzheimer's disease. *Frontiers in Cellular Neuroscience*, 2015, 9: 464.
111. GUILLOZET-BONGAARTS, A. L., et al. Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. *Neurobiology of Aging*, 2005, 26.7: 1015-1022.
112. GUO, H., et al. Active caspase-6 and caspase-6-cleaved tau in neuropil threads, neuritic plaques, and neurofibrillary tangles of Alzheimer's disease. *The American Journal of Pathology*, 2004, 165.2: 523-531.

113. GUPTA, S., et al. Selective interaction of JNK protein kinase isoforms with transcription factors. *The EMBO Journal*, 1996, 15.11: 2760.
114. HANES, J., et al. Rat tau proteome consists of six tau isoforms: implication for animal models of human tauopathies. *Journal of Neurochemistry*, 2009, 108.5: 1167-1176.
115. HANGER, D. P., et al. New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *Journal of Neurochemistry*, 1998, 71.6: 2465-2476.
116. HANGER, D. P., et al. Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *Journal of Biological Chemistry*, 2007, 282.32: 23645-23654.
117. HANGER, D. P.; ANDERTON, B. H.; NOBLE, W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends in Molecular Medicine*, 2009, 15.3: 112-119.
118. HANGER, D. P.; NOBLE, W. Functional implications of glycogen synthase kinase-3-mediated tau phosphorylation. *International Journal of Alzheimer's disease*, 2011, 2011: 352805.
119. HARDY, J. A.; HIGGINS, G. A. Alzheimer's disease: the amyloid cascade hypothesis. *Science*, 1992, 256.5054: 184.
120. HARPER, S. J.; WILKIE, N. MAPKs: new targets for neurodegeneration. *Expert Opinion on Therapeutic Targets*, 2003, 7.2: 187-200.
121. HARRINGTON, C. R., et al. Measurement of distinct immunochemical presentations of tau protein in Alzheimer disease. *Proceedings of the National Academy of Sciences*, 1991, 88.13: 5842-5846.
122. HARWOOD, A. J. Regulation of GSK-3: a cellular multiprocessor. *Cell*, 2001, 105.7: 821-824.
123. HENSLEY, K., et al. p38 kinase is activated in the Alzheimer's disease brain. *Journal of Neurochemistry*, 1999, 72.5: 2053-2058.
124. HERMANSON, G. T., Bioconjugate Techniques, Academic Press, New York 2013, pp. 227–228.
125. HIROKAWA, N.; SHIOMURA, Y.; OKABE, S. Tau proteins: the molecular structure and mode of binding on microtubules. *The Journal of Cell Biology*, 1988, 107.4: 1449-1459.
126. HIROKAWA, N., et al. Selective stabilization of tau in axons and microtubule-associated protein 2C in cell bodies and dendrites contributes to polarized localization of cytoskeletal proteins in mature neurons. *The Journal of Cell Biology*, 1996, 132.4: 667-679.
127. HO, G. J., et al. Altered p59 Fyn kinase expression accompanies disease progression in Alzheimer's disease: implications for its functional role. *Neurobiology of Aging*, 2005, 26.5: 625-635.
128. HOOPER, C.; KILLICK, R.; LOVESTONE, S. The GSK3 hypothesis of Alzheimer's disease. *Journal of Neurochemistry*, 2008, 104.6: 1433-1439.
129. HOROWITZ, P. M., et al. Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease. *Journal of Neuroscience*, 2004, 24.36: 7895-7902.
130. HROMADKOVA, L., et al. Identification and characterization of natural antibodies against tau protein in an intravenous immunoglobulin product. *Journal of Neuroimmunology*, 2015, 289: 121-129.
131. HROMADKOVA, L., et al. Difficulties associated with the structural analysis of proteins susceptible to form aggregates: The case of Tau protein as a biomarker of Alzheimer's disease. *Journal of Separation Science*, 2016, 39.4: 799-807.

132. HROMADKOVA, L., et al. Kinase-loaded magnetic beads for sequential in vitro phosphorylation of peptides and proteins. *Analyst*, 2018, 143.2: 466-474.
133. HYMAN, B. T. Caspase activation without apoptosis: insight into A [beta] initiation of neurodegeneration. *Nature Neuroscience*, 2011, 14.1: 5-6.
134. HYMAN, B. T.; YUAN, J. Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. *Nature Reviews Neuroscience*, 2012, 13.6: 395-406.
135. IHARA, Y., et al. Phosphorylated tau protein is integrated into paired helical filaments in Alzheimer's disease. *The Journal of Biochemistry*, 1986, 99.6: 1807-1810.
136. IKEZU, S.; IKEZU, T. Tau-tubulin kinase. *Frontiers in Molecular Neuroscience*, 2014, 7.
137. ILIFF, J. J., et al. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. *Journal of Neuroscience*, 2014, 34.49: 16180-16193.
138. IMAHORI, K.; UCHIDA, T. Physiology and pathology of tau protein kinases in relation to Alzheimer's disease. *The Journal of Biochemistry*, 1997, 121.2: 179-188.
139. ITTNER, L. M.; GÖTZ, J. Amyloid-β and tau—a toxic pas de deux in Alzheimer's disease. *Nature Reviews Neuroscience*, 2011, 12.2: 67.
140. ITZHAKI, R. F., et al. Microbes and Alzheimer's disease. *Journal of Alzheimer's disease*, 2016, 51.4: 979.
141. JACK, J. R., et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *The Lancet Neurology*, 2010, 9.1: 119-128.
142. JAKES, R., et al. Identification of 3-and 4-repeat tau isoforms within the PHF in Alzheimer's disease. *The EMBO Journal*, 1991, 10.10: 2725.
143. JANKOVICOVA, B., et al. Epitope mapping of allergen ovalbumin using biofunctionalized magnetic beads packed in microfluidic channels: The first step towards epitope-based vaccines. *Journal of Chromatography A*, 2008, 1206.1: 64-71.
144. JANKOVICOVA, B.arbora, et al. Quality evaluation of monoclonal antibodies suitable for immunomagnetic purification of native tau protein. *Scientific Papers of the University of Pardubice Series A, Faculty of Chemical Technology*, 2014, 20: 147-163.
145. JANKOVICOVA, B., et al. Benefits of Immunomagnetic Separation for Epitope Identification in Clinically Important Protein Antigens: A Case Study Using Ovalbumin, Carbonic Anhydrase I and Tau Protein. *Universal Journal of Biomedical Engineering*, 2015, 3: 1-8.
146. JEGANATHAN, S., et al. Global hairpin folding of tau in solution. *Biochemistry*, 2006, 45.7: 2283-2293.
147. JEYARAJAH, S., et al. Matrix-assisted laser desorption ionization/mass spectrometry mapping of human immunodeficiency virus-gp120 epitopes recognized by a limited polyclonal antibody. *Journal of the American Society for Mass Spectrometry*, 1998, 9.2: 157-165.
148. JICHA, G. A., et al. Alz-50 and MC-1, a new monoclonal antibody raised to paired helical filaments, recognize conformational epitopes on recombinant tau. *Journal of Neuroscience Research*, 1997, 48.2: 128-132.
149. JICHA, G. A.; BERENFELD, B.; DAVIES, P. Sequence requirements for formation of conformational variants of tau similar to those found in Alzheimer's disease. *Journal of Neuroscience Research*, 1999, 55.6: 713-723.
150. JICHA, G. A., et al. cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease. *Journal of Neuroscience*, 1999, 19.17: 7486-7494.
151. JOHNSON, G. V. W.; STOOTHOFF, W. H. Tau phosphorylation in neuronal cell function and dysfunction. *Journal of Cell Science*, 2004, 117.24: 5721-5729.

152. KADAVATH, H., et al. Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. *Proceedings of the National Academy of Sciences*, 2015, 112.24: 7501-7506.
153. KANAI, Y.; HIROKAWA, N. Sorting mechanisms of tau and MAP2 in neurons: suppressed axonal transit of MAP2 and locally regulated microtubule binding. *Neuron*, 1995, 14.2: 421-432.
154. KARSTEN, S. L., et al. A genomic screen for modifiers of tauopathy identifies puromycin-sensitive aminopeptidase as an inhibitor of tau-induced neurodegeneration. *Neuron*, 2006, 51.5: 549-560.
155. KAWAMATA, T., et al. A protein kinase, PKN, accumulates in Alzheimer neurofibrillary tangles and associated endoplasmic reticulum-derived vesicles and phosphorylates tau protein. *Journal of Neuroscience*, 1998, 18.18: 7402-7410.
156. KELLER, J. N.; HANNI, K. B.; MARKESBERY, W. R. Impaired proteasome function in Alzheimer's disease. *Journal of Neurochemistry*, 2000, 75.1: 436-439.
157. KEMPF, M., et al. Tau binds to the distal axon early in development of polarity in a microtubule-and microfilament-dependent manner. *Journal of Neuroscience*, 1996, 16.18: 5583-5592.
158. KENESSEY, A., et al. Degradation of tau by lysosomal enzyme cathepsin D: implication for Alzheimer neurofibrillary degeneration. *Journal of Neurochemistry*, 1997, 69.5: 2026-2038.
159. KENTRUP, H., et al. Dyrk, a dual specificity protein kinase with unique structural features whose activity is dependent on tyrosine residues between subdomains VII and VIII. *Journal of Biological Chemistry*, 1996, 271.7: 3488-3495.
160. KLEIN, C., et al. Process outgrowth of oligodendrocytes is promoted by interaction of fyn kinase with the cytoskeletal protein tau. *Journal of Neuroscience*, 2002, 22.3: 698-707.
161. KIDD, M. Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature*, 1963, 197.4863: 192-193.
162. KIEBER-EMMONS, T., et al. The promise of the anti-idiotype concept. *Frontiers in Oncology*, 2012, 2: 196.
163. KIMURA, R., et al. The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between β-amyloid production and tau phosphorylation in Alzheimer disease. *Human Molecular Genetics*, 2006, 16.1: 15-23.
164. KLAVER, A. C., et al. Specific serum antibody binding to phosphorylated and non-phosphorylated tau in non-cognitively impaired, mildly cognitively impaired, and Alzheimer's disease subjects: an exploratory study. *Translational Neurodegeneration*, 2017, 6.1: 32.
165. KOLAROVA, M., et al. Tau oligomers in sera of patients with Alzheimer's disease and aged controls. *Journal of Alzheimer's Disease*, 2017, 58.2: 471-478.
166. KONTAXI, C.; PICCARDO, P.; GILL, A. C. Lysine-Directed Post-translational Modifications of Tau Protein in Alzheimer's Disease and Related Tauopathies. *Frontiers in Molecular Biosciences*, 2017, 4: 56.
167. KONZACK, S., et al. Swimming against the tide: mobility of the microtubule-associated protein tau in neurons. *Journal of Neuroscience*, 2007, 27.37: 9916-9927.
168. KOPKE, E., et al. Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. *Journal of Biological Chemistry*, 1993, 268.32: 24374-24384.

169. KOSIK, K. S.; JOACHIM, C. L.; SELKOE, D. J. Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. *Proceedings of the National Academy of Sciences*, 1986, 83.11: 4044-4048.
170. KOVACS, G. G. Invited review: neuropathology of tauopathies: principles and practice. *Neuropathology and Applied Neurobiology*, 2015, 41.1: 3-23.
171. KRESTOVA, M., et al. Characterization of isolated tau-reactive antibodies from the IVIG product, plasma of patients with Alzheimer's disease and cognitively normal individuals. *Journal of Neuroimmunology*, 2017, 313: 16-24. **a**
172. KRESTOVA, M.; HROMADKOVA, L.; RICNY, J. Purification of Natural Antibodies against Tau Protein by Affinity Chromatography. *Natural Antibodies: Methods and Protocols*, 2017, 33-44. **b**
173. KSIEZAK-REDING, H., et al. Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 2003, 1639.3: 159-168.
174. KUCEROVA, J., et al. PEGylation of magnetic poly (glycidyl methacrylate) microparticles for microfluidic bioassays. *Materials Science and Engineering: C*, 2014, 40: 308-315.
175. KUDO, L. C., et al. Puromycin-sensitive aminopeptidase (PSA/NPEPPS) impedes development of neuropathology in hPSA/TAUP301L double-transgenic mice. *Human Molecular Genetics*, 2011, 20.9: 1820-1833.
176. KUHN, I., et al. Serum titers of autoantibodies against α-synuclein and tau in child-and adulthood. *Journal of Neuroimmunology*, 2018, 315: 33-39.
177. LAWS, S. M., et al. Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription. *Journal of Neurochemistry*, 2003, 84.6: 1215-1236.
178. LAZEBNIK, Y. A., et al. Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, 1994, 371.6495: 346-347.
179. LEBOUVIER, T., et al. The microtubule-associated protein tau is phosphorylated by Syk. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2008, 1783.2: 188-192.
180. LEBOUVIER, T.; PASQUIER, F.; BUÉE, L. Update on tauopathies. *Current Opinion in Neurology*, 2017, 30.6: 589-598.
181. LEE, G.; COWAN, N.; KIRSCHNER, M. The primary structure and heterogeneity of tau protein from mouse brain. *Science*, 1988, 239.4837: 285-288.
182. LEE, G., et al. Tau interacts with src-family non-receptor tyrosine kinases. *Journal of Cell Science*, 1998, 111.21: 3167-3177.
183. LEE, G., et al. Phosphorylation of tau by fyn: implications for Alzheimer's disease. *Journal of Neuroscience*, 2004, 24.9: 2304.
184. LEE, M.-S., et al. Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature*, 2000, 405.6784: 360-364.
185. LEE, M. J.; LEE, J. H.; RUBINSZTEIN, D. C. Tau degradation: the ubiquitin–proteasome system versus the autophagy-lysosome system. *Progress in Neurobiology*, 2013, 105: 49-59.
186. LEJTENYI, D.; MAZER, B. Consistency of protective antibody levels across lots of intravenous immunoglobulin preparations. *Journal of Allergy and Clinical Immunology*, 2008, 121.1: 254-255.
187. LEROY, K.; YILMAZ, Z.; BRION, J.-P. Increased level of active GSK-3β in Alzheimer's disease and accumulation in argyrophilic grains and in neurones at different stages of neurofibrillary degeneration. *Neuropathology and Applied Neurobiology*, 2007, 33.1: 43-55.

188. LEVIN, E. C., et al. Brain-reactive autoantibodies are nearly ubiquitous in human sera and may be linked to pathology in the context of blood–brain barrier breakdown. *Brain research*, 2010, 1345: 221-232.
189. LEW, J., et al. A brain-specific activator of cyclin-dependent kinase 5. *Nature*, 1994, 371.6496: 423-426.
190. LI, G.; YIN, H.; KURET, J. Casein kinase 1 delta phosphorylates tau and disrupts its binding to microtubules. *Journal of Biological Chemistry*, 2004, 279.16: 15938-15945.
191. LI, W.; LEE, V. M.-Y. Characterization of two VQIXXK motifs for tau fibrillization in vitro. *Biochemistry*, 2006, 45.51: 15692-15701.
192. LI, Y., et al. Screening of inhibitors of glycogen synthase kinase-3β from traditional Chinese medicines using enzyme-immobilized magnetic beads combined with high-performance liquid chromatography. *Journal of Chromatography A*, 2015, 1425: 8-16.
193. LINDWALL, G.; COLE, R. D. Phosphorylation affects the ability of tau protein to promote microtubule assembly. *Journal of Biological Chemistry*, 1984, 259.8: 5301-5305.
194. LITMAN, P., et al. Subcellular localization of tau mRNA in differentiating neuronal cell culture: implications for neuronal polarity. *Neuron*, 1993, 10.4: 627-638.
195. LIU, C.; GÖTZ, J. Profiling murine tau with 0N, 1N and 2N isoform-specific antibodies in brain and peripheral organs reveals distinct subcellular localization, with the 1N isoform being enriched in the nucleus. *PLoS One*, 2013, 8.12: e84849.
196. LIU, F., et al. Involvement of aberrant glycosylation in phosphorylation of tau by cdk5 and GSK-3β. *FEBS Letters*, 2002, 530.1-3: 209-214.
197. LIU, F., et al. Dephosphorylation of tau by protein phosphatase 5 impairment in Alzheimer's disease. *Journal of Biological Chemistry*, 2005, 280.3: 1790-1796. **a**
198. LIU, F., et al. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *European Journal of Neuroscience*, 2005, 22.8: 1942-1950. **b**
199. LIU, F, et al. Truncation and activation of calcineurin A by calpain I in Alzheimer disease brain. *Journal of Biological Chemistry*, 2005, 280.45: 37755-37762. **c**
200. LIU, F.; LIANG, Z.; GONG, C. X. Hyperphosphorylation of tau and protein phosphatases in Alzheimer disease. *Panminerva medica*, 2006, 48.2: 97.
201. LIU, F., et al. PKA modulates GSK-3β-and cdk5-catalyzed phosphorylation of tau in site-and kinase-specific manners. *FEBS Letters*, 2006, 580.26: 6269-6274.
202. LIU, S. J., et al. Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain. *Journal of Biological Chemistry*, 2004, 279.48: 50078-50088.
203. LLEO, A., et al. Definition of human autoimmunity—autoantibodies versus autoimmune disease. *Autoimmunity Reviews*, 2010, 9.5: A259-A266.
204. LLORENS-MARÍTIN, M., et al. GSK-3β, a pivotal kinase in Alzheimer disease. *Frontiers in Molecular Neuroscience*, 2014, 7.
205. LOEFFLER, D. A. Should development of Alzheimer's disease-specific intravenous immunoglobulin be considered?. *Journal of Neuroinflammation*, 2014, 11.1: 198.
206. LOEFFLER, D. A.; KLAVER, A. C.; COFFEY, M. P. ELISA measurement of specific antibodies to phosphorylated tau in intravenous immunoglobulin products. *International Immunopharmacology*, 2015, 28.2: 1108-1112.
207. LOEFFLER, D. A.; KLAVER, A. C. Polyvalent immunoglobulin binding is an obstacle to accurate measurement of specific antibodies with ELISA despite inclusion of blocking agents. *International Immunopharmacology*, 2017, 52: 227-229.

208. LOOMIS, P. A., et al. Identification of nuclear tau isoforms in human neuroblastoma cells. *Proceedings of the National Academy of Sciences*, 1990, 87.21: 8422-8426.
209. LOPRESTI, P., et al. Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. *Proceedings of the National Academy of Sciences*, 1995, 92.22: 10369-10373.
210. LUND, H., et al. Tau-Tubulin Kinase 1 Expression, Phosphorylation and Co-Localization with Phospho-Ser422 Tau in the Alzheimer's Disease Brain. *Brain Pathology*, 2013, 23.4: 378-389.
211. LYE, T. C.; SHORES, E. A. Traumatic brain injury as a risk factor for Alzheimer's disease: a review. *Neuropsychology Review*, 2000, 10.2: 115-129.
212. MACCIONI, R. B., et al. The revitalized tau hypothesis on Alzheimer's disease. *Archives of Medical Research*, 2010, 41.3: 226-231.
213. MAGI, S., et al. Intracellular calcium dysregulation: Implications for Alzheimer's disease. *BioMed Research International*, 2016, 2016.
214. MALMQVIST, T.; ANTHONY, K.; GALLO, J.-M. Tau mRNA is present in axonal RNA granules and is associated with elongation factor 1A. *Brain Research*, 2014, 1584: 22-27.
215. MANDELKOW, E.-M.; MANDELKOW, E. Biochemistry and cell biology of tau protein in neurofibrillary degeneration. *Cold Spring Harbor Perspectives in Medicine*, 2012, 2.7: a006247.
216. MANNING, G., et al. The protein kinase complement of the human genome. *Science*, 2002, 298.5600: 1912-1934.
217. MAQBOOL, M.; MOBASHIR, M.; HODA, N. Pivotal role of glycogen synthase kinase-3: A therapeutic target for Alzheimer's disease. *European Journal of Medicinal Chemistry*, 2016, 107: 63-81.
218. MARTIN, L.; LATYPOVA, X.; TERRO, F. Post-translational modifications of tau protein: implications for Alzheimer's disease. *Neurochemistry International*, 2011, 58.4: 458-471.
219. MARTIN, L., et al. Tau protein kinases: involvement in Alzheimer's disease. *Ageing Research Reviews*, 2013, 12.1: 289-309.
220. MARUSZAK, A., et al. Hippocampal neurogenesis in Alzheimer's disease: is there a role for dietary modulation?. *Journal of Alzheimer's Disease*, 2014, 38.1: 11-38.
221. MATENIA, D.; MANDELKOW, E.-M. The tau of MARK: a polarized view of the cytoskeleton. *Trends in Biochemical Sciences*, 2009, 34.7: 332-342.
222. MCLELLAN, S., et al. Studies on the tissue distribution of the puromycin-sensitive enkephalin-degrading aminopeptidases. *Journal of Neurochemistry*, 1988, 51.5: 1552-1559.
223. MEAD, E., et al. Halting of Caspase Activity Protects Tau from MC1-Conformational Change and Aggregation. *Journal of Alzheimer's Disease*, 2016, 54.4: 1521-1538.
224. MENA, R., et al. A progressive deposition of paired helical filaments (PHF) in the brain characterizes the evolution of dementia in Alzheimer's disease. An immunocytochemical study with a monoclonal antibody against the PHF core. *Journal of Neuropathology & Experimental Neurology*, 1991, 50.4: 474-490.
225. MENA, R., et al. Staging the pathological assembly of truncated tau protein into paired helical filaments in Alzheimer's disease. *Acta Neuropathologica*, 1996, 91.6: 633-641.
226. MINEGISHI, S., et al. Membrane association facilitates degradation and cleavage of the cyclin-dependent kinase 5 activators p35 and p39. *Biochemistry*, 2010, 49.26: 5482-5493.
227. MONDRAGON-RODRIGUEZ, S., et al. Cleavage and conformational changes of tau protein follow phosphorylation during Alzheimer's disease. *International Journal of Experimental Pathology*, 2008, 89.2: 81-90.

228. MONDRAGON-RODRIGUEZ, S., et al. Interaction of endogenous tau protein with synaptic proteins is regulated by N-methyl-D-aspartate receptor-dependent tau phosphorylation. *Journal of Biological Chemistry*, 2012, 287.38: 32040-32053.
229. MORISHIMA-KAWASHIMA, M., et al. Proline-directed and non-proline-directed phosphorylation of PHF-tau. *Journal of Biological Chemistry*, 1995, 270.2: 823-829.
230. MORRIS, M., et al. Tau post-translational modifications in wild-type and human amyloid precursor protein transgenic mice. *Nature Neuroscience*, 2015, 18.8: 1183.
231. MUKRASCH, M. D., et al. Structural polymorphism of 441-residue tau at single residue resolution. *PLoS Biology*, 2009, 7.2: e1000034.
232. MULLER, R., et al. Expression of microtubule-associated proteins MAP2 and tau in cultured rat brain oligodendrocytes. *Cell and Tissue Research*, 1997, 288.2: 239-249.
233. NELSON, P. T., et al. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. *Journal of Neuropathology & Experimental Neurology*, 2012, 71.5: 362-381.
234. NEVE, R. L., et al. Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Molecular Brain Research*, 1986, 1.3: 271-280.
235. NICHOLLS, S. B., et al. Characterization of TauC3 antibody and demonstration of its potential to block tau propagation. *PloS One*, 2017, 12.5: e0177914.
236. NIXON, R. A.; YANG, D.-S. Autophagy failure in Alzheimer's disease - locating the primary defect. *Neurobiology of Disease*, 2011, 43.1: 38-45.
237. NOBLE, W., et al. The importance of tau phosphorylation for neurodegenerative diseases. *Frontiers in Neurology*, 2013, 4.
238. NOVAK, M.; KABAT, J.; WISCHIK, C. M. Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. *The EMBO Journal*, 1993, 12.1: 365.
239. OCHSENBEIN, A. F.; ZINKERNAGEL, R. M. Natural antibodies and complement link innate and acquired immunity. *Immunology Today*, 2000, 21.12: 624-630.
240. OKUYAMA, K., et al. Linkage-dependent contribution of repeat peptides to self-aggregation of three-or four-repeat microtubule-binding domains in tau protein. *The FEBS Journal*, 2008, 275.7: 1529-1539.
241. OLESEN, O. F. Proteolytic Degradation of Microtubule-Associated Protein τ by Thrombin. *Biochemical and Biophysical Research Communications*, 1994, 201.2: 716-721.
242. OMRI, B., et al. The Lck tyrosine kinase is expressed in brain neurons. *Journal of Neurochemistry*, 1996, 67.4: 1360-1364.
243. PALLAS-BAZARRA, N., et al. Novel function of Tau in regulating the effects of external stimuli on adult hippocampal neurogenesis. *The EMBO Journal*, 2016, 35.13: 1417-1436.
244. PANDA, S.; DING, J. L. Natural antibodies bridge innate and adaptive immunity. *The journal of Immunology*, 2015, 194.1: 13-20.
245. PARASCHIV, G., et al. Epitope structure and binding affinity of single chain llama anti-β-amyloid antibodies revealed by proteolytic excision affinity-mass spectrometry. *Journal of Molecular Recognition*, 2013, 26.1: 1-9.
246. PARK, S.-Y.; FERREIRA, A. The generation of a 17 kDa neurotoxic fragment: an alternative mechanism by which tau mediates β-amyloid-induced neurodegeneration. *Journal of Neuroscience*, 2005, 25.22: 5365-5375.

247. PARK, J.; SONG, W.-J.; CHUNG, K. C. Function and regulation of Dyrk1A: towards understanding Down syndrome. *Cellular and Molecular Life Sciences*, 2009, 66.20: 3235-3240.
248. PAUDEL, H. K., et al. Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. *Journal of Biological Chemistry*, 1993, 268.31: 23512-23518.
249. PAUDEL, H. K. The regulatory Ser262 of microtubule-associated protein tau is phosphorylated by phosphorylase kinase. *Journal of Biological Chemistry*, 1997, 272.3: 1777-1785.
250. PATRICK, G. N., et al. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature*, 1999, 402.6762: 615-622.
251. PEARCE, L. R.; KOMANDER, D.; ALESSI, D. R. The nuts and bolts of AGC protein kinases. *Nature reviews Molecular Cell Biology*, 2010, 11.1: 9-22.
252. PEDERSEN, J. T.; SIGURDSSON, E. M. Tau immunotherapy for Alzheimer's disease. *Trends in Molecular Medicine*, 2015, 21.6: 394-402.
253. PEI, J.-J., et al. Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. *Journal of Alzheimer's Disease*, 2001, 3.1: 41-48.
254. PENG, I.; BINDER, L. I.; BLACK, M. M. Biochemical and immunological analyses of cytoskeletal domains of neurons. *The Journal of Cell Biology*, 1986, 102.1: 252-262.
255. PEVALOVA, M., et al. Post-translational modifications of tau protein. *Bratislavske Lekarske Listy*, 2006, 107.9-10: 346-353.
256. PIZZI, M., et al. Differential expression of fetal and mature tau isoforms in primary cultures of rat cerebellar granule cells during differentiation in vitro. *Molecular Brain Research*, 1995, 34.1: 38-44.
257. RAMCHARITAR, J., et al. Cerebrospinal fluid tau cleaved by caspase-6 reflects brain levels and cognition in aging and Alzheimer disease. *Journal of Neuropathology & Experimental Neurology*, 2013, 72.9: 824-832.
258. RAO, M. V., et al. Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. *Journal of Neuroscience*, 2008, 28.47: 12241-12254.
259. REGAN, P.; WHITCOMB, D. J.; CHO, K. Physiological and pathophysiological implications of synaptic tau. *The Neuroscientist*, 2017, 23.2: 137-151.
260. REINECKE, J. B., et al. Implicating calpain in tau-mediated toxicity in vivo. *PLoS One*, 2011, 6.8: e23865.
261. RELKIN, N. Clinical trials of intravenous immunoglobulin for Alzheimer's disease. *Journal of Clinical Immunology*, 2014, 34.1: 74-79.
262. RELKIN, N. R., et al. A phase 3 trial of IV immunoglobulin for Alzheimer disease. *Neurology*, 2017, 88.18: 1768-1775.
263. REYNOLDS, C. H., et al. Phosphorylation sites on tau identified by nanoelectrospray mass spectrometry. *Journal of Neurochemistry*, 2000, 74.4: 1587-1595.
264. REYNOLDS, C. H., et al. Phosphorylation regulates tau interactions with Src homology 3 domains of phosphatidylinositol 3-kinase, phospholipase Cγ1, Grb2, and Src family kinases. *Journal of Biological Chemistry*, 2008, 283.26: 18177-18186.
265. RISSMAN, R. A., et al. Caspase-cleavage of tau is an early event in Alzheimer disease tangle pathology. *Journal of Clinical Investigation*, 2004, 114.1: 121.

266. ROHN, T. T., et al. Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *The American Journal of Pathology*, 2001, 158.1: 189-198.
267. ROHN, T. T., et al. Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain. *Neurobiology of Disease*, 2002, 11.2: 341-354.
268. ROSENMANN, H., et al. Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. *Neuroscience Letters*, 2006, 410.2: 90-93.
269. ROSSI, F.; DIETRICH, G.; KAZATCHKINE, M. D. Anti-idiotypes against Autoantibodies in Normal Immunoglobulins: Evidence for Network Regulation of Human Autoimmune Responses. *Immunological Reviews*, 1989, 110.1: 135-149.
270. ROSSI, Giacomina, et al. A new function of microtubule-associated protein tau: involvement in chromosome stability. *Cell Cycle*, 2008, 7.12: 1788-1794.
271. ROUX, P. P.; BLENIS, J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and Molecular Biology Reviews*, 2004, 68.2: 320-344.
272. SAITO, K. l., et al. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proceedings of the National Academy of Sciences*, 1993, 90.7: 2628-2632.
273. SATO, S., et al. Tau-tubulin kinase 1 (TTBK1), a neuron-specific tau kinase candidate, is involved in tau phosphorylation and aggregation. *Journal of Neurochemistry*, 2006, 98.5: 1573-1584.
274. SATO, S., et al. Spatial learning impairment, enhanced CDK5/p35 activity, and downregulation of NMDA receptor expression in transgenic mice expressing tau-tubulin kinase 1. *Journal of Neuroscience*, 2008, 28.53: 14511-14521.
275. SCALES, T. M. E., et al. Tyrosine phosphorylation of tau by the SRC family kinases lck and fyn. *Molecular Neurodegeneration*, 2011, 6.1: 12.
276. SCHNEIDER, A., et al. Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. *Biochemistry*, 1999, 38.12: 3549-3558.
277. SCHOLZ, T.; MANDELKOW, E. Transport and diffusion of Tau protein in neurons. *Cellular and Molecular Life Sciences*, 2014, 71.16: 3139-3150.
278. SCHROEDER, S. K., et al. Tau-directed immunotherapy: a promising strategy for treating Alzheimer's disease and other tauopathies. *Journal of Neuroimmune Pharmacology*, 2016, 11.1: 9-25.
279. SCHWAB, C., et al. Casein kinase 1 delta is associated with pathological accumulation of tau in several neurodegenerative diseases. *Neurobiology of Aging*, 2000, 21.4: 503-510.
280. SCHWEERS, O., et al. Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure. *Journal of Biological Chemistry*, 1994, 269.39: 24290-24297.
281. SCOFIELD, R. H. Autoantibodies as predictors of disease. *The Lancet*, 2004, 363.9420: 1544-1546.
282. SELDEN, S. C.; POLLARD, T. D. Phosphorylation of microtubule-associated proteins regulates their interaction with actin filaments. *Journal of Biological Chemistry*, 1983, 258.11: 7064-7071.
283. SELKOE, D. J.; HARDY, J. The amyloid hypothesis of Alzheimer9s disease at 25 years. *EMBO Molecular Medicine*, 2016, 8.6: 595-608.

284. SENGUPTA, A., et al. Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. *Archives of Biochemistry and Biophysics*, 1998, 357.2: 299-309.
285. SENGUPTA, S., et al. Degradation of tau protein by puromycin-sensitive aminopeptidase in vitro. *Biochemistry*, 2006, 45.50: 15111-15119.
286. SERGEANT, N., et al. Biochemistry of Tau in Alzheimer's disease and related neurological disorders. *Expert Review of Proteomics*, 2008, 5.2: 207-224.
287. SERRANO-POZO, A., et al. Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, 2011, 1.1: a006189.
288. SHABB, J. B. Physiological substrates of cAMP-dependent protein kinase. *Chemical Reviews*, 2001, 101.8: 2381-2412.
289. SHIRAZI, S. K.; WOOD, John G. The protein tyrosine kinase, fyn, in Alzheimer's disease pathology. *Neuroreport*, 1993, 4.4: 435-437.
290. SINGH, T. J., et al. Calcium/calmodulin-dependent protein kinase II phosphorylates tau at Ser-262 but only partially inhibits its binding to microtubules. *FEBS Letters*, 1996, 387.2-3: 145-148.
291. SIRONI, J. J., et al. Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK. *FEBS Letters*, 1998, 436.3: 471-475.
292. SKRABANA, R., et al. Folding of Alzheimer's core PHF subunit revealed by monoclonal antibody 423. *FEBS Letters*, 2004, 568.1-3: 178-182.
293. SKRABANA, R.; SEVCIK, J.; NOVAK, M. Intrinsically disordered proteins in the neurodegenerative processes: formation of tau protein paired helical filaments and their analysis. *Cellular and Molecular Neurobiology*, 2006, 26.7-8: 1083-1095.
294. SMITH, L. M., et al. Intravenous immunoglobulin products contain specific antibodies to recombinant human tau protein. *International Immunopharmacology*, 2013, 16.4: 424-428.
295. SMITH, L. M.; COFFEY, M. P.; LOEFFLER, D. A. Specific binding of intravenous immunoglobulin products to tau peptide fragments. *International Immunopharmacology*, 2014, 21.2: 279-282.
296. SONTAG, E., et al. Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. *Neuron*, 1996, 17.6: 1201-1207.
297. SONTAG, E., et al. Altered expression levels of the protein phosphatase 2A ABαC enzyme are associated with Alzheimer disease pathology. *Journal of Neuropathology & Experimental Neurology*, 2004, 63.4: 287-301.
298. STEINER, B., et al. Phosphorylation of microtubule-associated protein tau: identification of the site for Ca2 (+)-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. *The EMBO Journal*, 1990, 9.11: 3539.
299. SU, J. H., et al. Activated caspase-3 expression in Alzheimer's and aged control brain: correlation with Alzheimer pathology. *Brain Research*, 2001, 898.2: 350-357.
300. SUGINO, E., et al. Three-/four-repeat-dependent aggregation profile of tau microtubule-binding domain clarified by dynamic light scattering analysis. *Biochemical and Biophysical Research Communications*, 2009, 385.2: 236-240.
301. SUN, X., et al. Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species. *Journal of Biological Chemistry*, 2000, 275.23: 17237-17240.
302. SWERDLOW, R. H.; KHAN, S. M. A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Medical Hypotheses*, 2004, 63.1: 8-20.
303. TAKUMA, H.; ARAWAKA, S.; MORI, H. Isoforms changes of tau protein during development in various species. *Developmental Brain Research*, 2003, 142.2: 121-127.

304. TARASOFF-CONWAY, J. M., et al. Clearance systems in the brain—implications for Alzheimer disease. *Nature Reviews Neurology*, 2015, 11.8: 457.
305. TATEBAYASHI, Y., et al. Role of tau phosphorylation by glycogen synthase kinase-3β in the regulation of organelle transport. *Journal of Cell Science*, 2004, 117.9: 1653-1663.
306. TAYLOR, S. S.; BUECHLER, J. A.; YONEMOTO, W. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry*, 1990, 59.1: 971-1005.
307. TERWEL, D., et al. Amyloid activates GSK-3β to aggravate neuronal tauopathy in bigenic mice. *The American Journal of Pathology*, 2008, 172.3: 786-798.
308. THOMAS, G. M.; HUGANIR, R. L. MAPK cascade signalling and synaptic plasticity. *Nature Reviews Neuroscience*, 2004, 5.3: 173-183.
309. TOMIZAWA, K., et al. Tau-tubulin kinase phosphorylates tau at Ser-208 and Ser-210, sites found in paired helical filament-tau. *Febs Letters*, 2001, 492.3: 221-227.
310. TREMBLAY, M. A.; ACKER, C. M.; DAVIES, P. Tau phosphorylated at tyrosine 394 is found in Alzheimer's disease tangles and can be a product of the Abl-related kinase, Arg. *Journal of Alzheimer's Disease*, 2010, 19.2: 721-733.
311. TRINCZEK, B., et al. Tau regulates the attachment/detachment but not the speed of motors in microtubule-dependent transport of single vesicles and organelles. *Journal of Cell Science*, 1999, 112.14: 2355-2367.
312. TROJANOWSKI, J. Q., et al. Localization of the mitogen activated protein kinase ERK2 in Alzheimer's disease neurofibrillary tangles and senile plaque neurites. *Brain Research*, 1993, 618.2: 333-337.
313. TSAI, L. H., et al. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development*, 1993, 119.4: 1029-1040.
314. UMEMORI, H., et al. Specific expressions of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. *Molecular Brain Research*, 1992, 16.3: 303-310.
315. VECHTEROVA, L., et al. DC11: a novel monoclonal antibody revealing Alzheimer's disease-specific tau epitope. *Neuroreport*, 2003, 14.1: 87-91.
316. VEGA, I. E., et al. Increase in tau tyrosine phosphorylation correlates with the formation of tau aggregates. *Molecular Brain Research*, 2005, 138.2: 135-144.
317. VENNE, A. S.; KOLLIPARA, L.; ZAHEDI, R. P. The next level of complexity: crosstalk of posttranslational modifications. *Proteomics*, 2014, 14.4-5: 513-524.
318. VIOLET, M., et al. A major role for Tau in neuronal DNA and RNA protection in vivo under physiological and hyperthermic conditions. *Frontiers in Cellular Neuroscience*, 2014, 8.
319. VON BERGEN, M., et al. Assembly of τ protein into Alzheimer paired helical filaments depends on a local sequence motif (306VQIVYK311) forming β structure. *Proceedings of the National Academy of Sciences*, 2000, 97.10: 5129-5134.
320. WANG, Y., et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. *Human Molecular Genetics*, 2009, 18.21: 4153-4170.
321. WANG, Y., et al. Proteolytic processing of tau. *Biochemical Society Transactions*, 2010, 38.4: 955-961.
322. WANG, Y.; MANDELKOW, E. Degradation of tau protein by autophagy and proteasomal pathways. *Biochemical Society Transactions*, 2012, 40.4: 644-652.
323. WANG, J.-Z., et al. Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. *Journal of Alzheimer's Disease*, 2013, 33.s1: S123-S139.

324. WANG, P.; WANG, Z.-Y. Metal ions influx is a double edged sword for the pathogenesis of Alzheimer's disease. *Ageing Research Reviews*, 2017, 35: 265-290.
325. WEGMANN, S., et al. Human Tau isoforms assemble into ribbon-like fibrils that display polymorphic structure and stability. *Journal of Biological Chemistry*, 2010, 285.35: 27302-27313.
326. WEINGARTEN, M. D., et al. A protein factor essential for microtubule assembly. *Proceedings of the National Academy of Sciences*, 1975, 72.5: 1858-1862.
327. WILLIS, J. R., et al. Human germline antibody gene segments encode polyspecific antibodies. *PLoS Computational Biology*, 2013, 9.4: e1003045.
328. WISCHIK, C. M., et al. Subunit structure of paired helical filaments in Alzheimer's disease. *The Journal of Cell Biology*, 1985, 100.6: 1905-1912.
329. WISCHIK, C. M., et al. Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. *Proceedings of the National Academy of Sciences*, 1988, 85.12: 4506-4510.
330. WISNIEWSKI, T.; GONI, F. Immunotherapeutic approaches for Alzheimer's disease. *Neuron*, 2015, 85.6: 1162-1176.
331. WOOD, J. G., et al. Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau (tau). *Proceedings of the National Academy of Sciences*, 1986, 83.11: 4040-4043.
332. WOODGETT, J. R. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *The EMBO Journal*, 1990, 9.8: 2431.
333. WOODGETT, J. R. Judging a protein by more than its name: GSK-3. *Science Signaling STKE*, 2001, 100: re12.
334. WOODS, Y. L., et al. The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bε at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. *Biochemical Journal*, 2001, 355.3: 609-615.
335. YAMAMOTO, H., et al. Dephosphorylation of microtubule proteins by brain protein phosphatases 1 and 2A, and its effect on microtubule assembly. *Journal of Neurochemistry*, 1988, 50.5: 1614-1623.
336. YANAGI, S., et al. Syk expression and novel function in a wide variety of tissues. *Biochemical and Biophysical Research Communications*, 2001, 288.3: 495-498.
337. YANG, X.-J.; SETO, E. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Molecular Cell*, 2008, 31.4: 449-461.
338. YASOJIMA, K., et al. Casein kinase 1 delta mRNA is upregulated in Alzheimer disease brain. *Brain Research*, 2000, 865.1: 116-120.
339. YIN, X., et al. Brain endothelial cells synthesize neurotoxic thrombin in Alzheimer's disease. *The American Journal of Pathology*, 2010, 176.4: 1600-1606.
340. YU, Y., et al. Developmental regulation of tau phosphorylation, tau kinases, and tau phosphatases. *Journal of Neurochemistry*, 2009, 108.6: 1480-1494.
341. ZEMPEL, H., et al. Axodendritic sorting and pathological missorting of Tau is isoform specific and determined by axon initial segment architecture. *Journal of Biological Chemistry*, 2017, 292, 29 :12192-12207.
342. ZETTERBERG, H., et al. Plasma tau levels in Alzheimer's disease. *Alzheimer's Research & Therapy*, 2013, 5.2: 9.

343. ZHANG, F., et al. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium Evidence for autoregulation of GSK-3. *Journal of Biological Chemistry*, 2003, 278.35: 33067-33077.
344. ZHANG, Z., et al. Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nature Medicine*, 2014, 20.11: 1254-1262.
345. ZHU, X., et al. Activation and redistribution of c-Jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. *Journal of Neurochemistry*, 2001, 76.2: 435-441.
346. ZILKA, N., et al. Truncated tau from sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo. *FEBS Letters*, 2006, 580.15: 3582-3588.
347. ZIVKOVIC, S. Intravenous immunoglobulin in the treatment of neurologic disorders. *Acta Neurologica Scandinavica*, 2016, 133.2: 84-96.