Effect of amyloid precursor protein and tau on dendritic spines and cell survival in an *ex vivo* model of Alzheimer's disease

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To Maria, Maya and Mio

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1. Abstract

Alzheimer's disease (AD) is characterized by progressive neuronal loss and synaptic deterioration as evidenced by a loss of dendritic spines. Histopathological hallmarks represent extracellular amyloid plaques, consisting of aggregated amyloid beta (Aβ) peptide, a cleavage product of the amyloid precursor protein (APP) and neurofibrillary tangles (NFTs) with hyperphosphorylated tau protein as major component.

Using Sindbis virus-mediated expression of tau and a hyperphosphorylation-mimicking tau mutant (PHP tau) in mouse organotypic hippocampal slice cultures, it is shown that the disease-relevant modification in PHP-tau induces pathological tau conformation and causes region-specific cell death. In contrast to the toxic activity, neuronal connectivity as evidenced by density and morphology of dendritic spines is remarkably stable against PHP tau-dependent degeneration.

To analyze the functional interaction of tau and Aβ with respect to spine alterations and cell death, hippocampal slice cultures from transgenic mice expressing mutated APP containing three familial AD mutations were prepared. These mice produce equimolar amounts of $A\beta_{40}$ and $A\beta_{42}$ already in embryonic age. Slices of APP transgenic mice and non-transgenic littermates were then infected with Sindbis virus expressing tau constructs. Confocal high-resolution imaging, algorithm-based evaluation of spine morphology and live imaging was used to determine spine changes and cell death. It is shown that Aβ induces spine loss and alters spine shape independent of tau expression. In contrast, Aβ alone does not cause neuronal degeneration but requires the presence of human tau to induce cell death through an increased tau phosphorylation. In agreement, levels of GSK-3β, a major tau kinase, are upregulated in APP transgenic cultures. Phosphorylation-blocking mutations in Ala tau prevent Aβ-induced tau toxicity, confirming the role of phosphorylation for mediating tau toxicity. Preventing Aβ production by blocking γ-secretase with γ-secretase inhibitor DAPT abolishes both, spine pathology and tau-dependent neurodegeneration indicating that Aβ pathology is upstream of tau pathology. Although both, spine alterations and cell death are mediated by NMDA glutamate receptors (NMDARs), the downstream cascades substantially differ. GSK-3β is activated and participates in both cascades, whereas calcineurin is only involved in mediating spine loss. Thus, we hypothesize that both cascades occur in different cellular compartments. Interestingly, Aβ also induces toxicity of the FTDP-17 tau mutant R406W tau but independent of GSK-3β.

2. Introduction

2.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder world-wide In Germany about 1,1 million people currently suffer from AD with 250000 new cases per year. Since AD is an age-related dementia and due to the massive aging of our society, the number of diseased people will increase to 2,6 million until 2050 (Deutsche Alzheimer Gesellschaft). Patients suffering from AD show cognitive decline as seen in difficulties with declarative memory and sense of direction, depressive symptoms, aphasia (language disorder), apraxia (loss of the ability to execute or carry out learned purposeful movements), behavioural changes and aggressiveness (Benke, 1993; Franke, 1994; Förstl and Kurz, 1999). Thus, finding appropriate therapies will be one of the major challenges within the next years.
AD was first characterized by the German physician Alois Alzheimer in 1907. His patient, Auguste D., a 51 year-old woman, showed symptoms unlike others he had seen before. Her answers to personal questions did not match (See the interview with Auguste D. in the Appendix). When Auguste D. died Alois Alzheimer analyzed her brain in the Royal psychiatric clinic in Munich. He realized a massive loss of brain mass. Within the remaining neurons he observed fibrillary structures and the cortex was full of plaques (Fig.1).

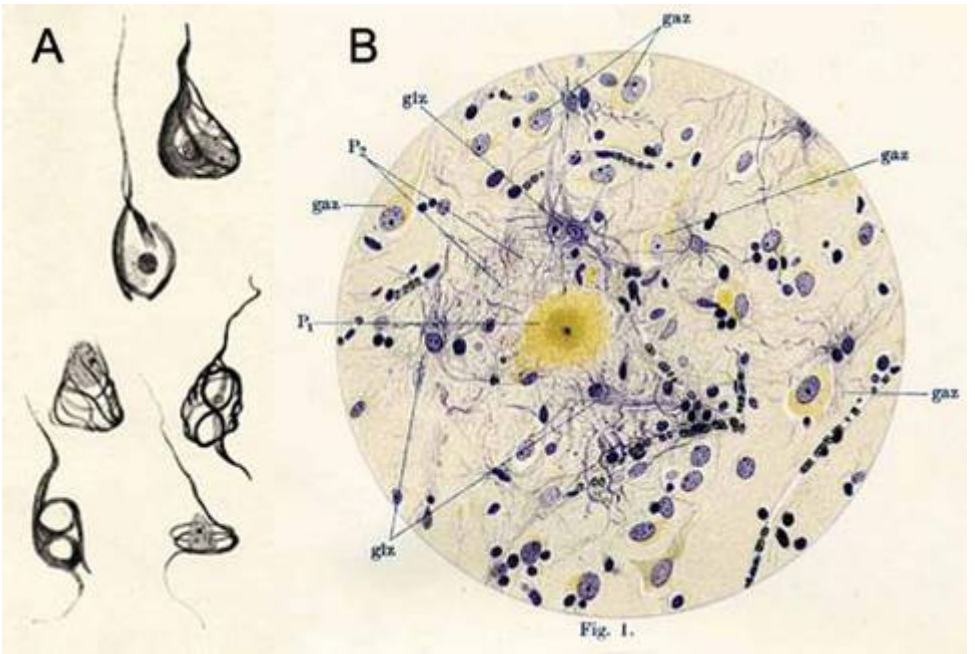

Fig. 1: Alois Alzheimer's drawings of (A) intracellular fibrils from Auguste D. and (B) a histological section from patient Johann F.
gaz: ganglia cell;
glz: glia cell;
P: extracellular plaque
(Dahm, 2009)

It took eight decades until the composition of these aggregates could be identified. Collin Masters and colleagues (1985) found amyloid-beta (Aβ), a cleavage product of the amyloid precursor protein APP, to be the major component of the extracellular (amyloid, senile) plaques. The intracellular fibrillary structures (neurofibrillary tangles (NFTs)) mainly contain hyperphosphorylated tau protein (Kosik et al., 1986).

AD is categorized in two different forms. Most incidences occur in elderly (> 65 years) as sporadic or late-onset AD with the apolipoprotein E4 allel as main risk factor. However, familial cases (familial Alzheimer's disease, FAD) with early-onset are found in people <65 years (Brouwers et al., 2008; Waring and Rosenberg, 2008). FAD cases occur due to autosomal dominant mutations in the APP or presenilin genes (PS1 or PS2) which cause a shift of APP cleavage to the most amyloidogenic form of Aβ, $A\beta_{42}$ (Haass and De Strooper, 1999).

2.2 Microtubule-associated protein tau

Structure and function of tau

Tau belongs to the family of microtubule-associated proteins (MAPs) and is mainly located in the axon of neurons (Binder et al., 1985). As a MAP tau is thought to play an important role in mediating the assembly and stabilization of the microtubule network which is the main structural determinant of the neuronal cell. The human tau gene is located on Chromosome 17 and consists of 16 exons. Due to alternative splicing of exons 2, 3 and 10 six isoforms are found in the human central nervous system (CNS) which are ranging from 352 to 441 amino acids (Fig. 2A). In the fetal brain only the smallest tau isoform is expressed whereas all are produced in the adult brain. Based on amino acid sequences tau can be divided into different regions (Fig. 2B). The aminoterminal domain projects from the microtubule after tau has bound (Fig. 2B right) and may be responsible for interaction with other cytoskeletal elements or the plasma membrane (Brandt et al., 1995). The proline-rich region contains several phosphorylation sites and contains an additional weak microtubule-binding site (Brandt and Lee, 1993). The repeat region containing either three or four repeat-domains - dependent on the insertion of Exon 10 - comprises the main microtubule interaction site of tau (Lee et al., 1989) since several positive charged amino acids facilitate the binding to negatively charged microtubules.

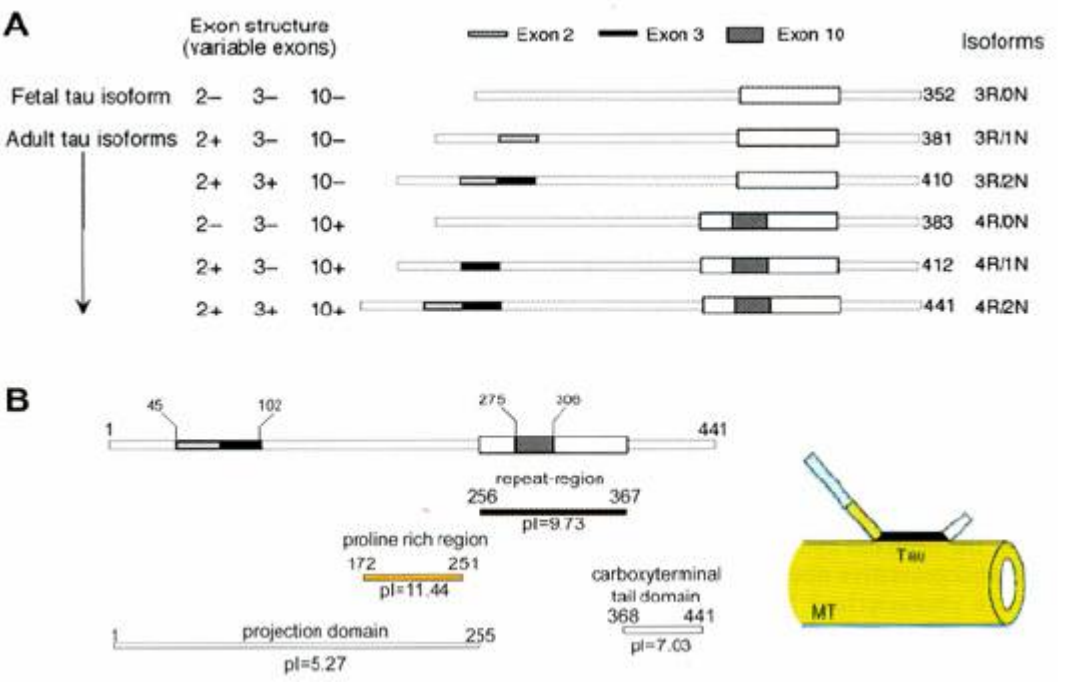

Fig. 2: Schematic representation of tau structure. **(A)** Six different tau isoforms are found in the human central nervous system (CNS). **(B)** Molecular and functional organization of tau shown for the longest CNS isoform, based on amino acid composition and sequence features. (Brandt and Leschik, 2004)

The interaction of tau with microtubules is regulated by phosphorylation. Tau isolated from brains of healthy people is phosphorylated at several sited. Several tau kinases such as GSK3β, MAPK, cdk5, MARK of PKA could be shown to phosphorylate tau (reviewed in Buée et al., 2000). Tau phosphorylation can induce conformational changes in tau, reduce its ability to promote de novo nucleation of microtubules, decrease its affinity to microtubules and therefore increase dynamic instability of microtubules (Hagestedt et al., 1989; Drechsel et al., 1992; Biernat et al., 1993; Brandt et al., 1994).

Tau in diseases

In 1986 first evidence for a role of tau in Alzheimer's disease came up when it was identified to be the major component of NFTs (Kosik et al., 1986). Nowadays, several diseases, called tauopathies, are known in which tau forms insoluble aggregates. The most common types of tauopathies are AD, Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease and progressive supranuclear palsy. Some tauopathies but not AD can be caused by mutations in the tau gene, which induce abnormal tau phosphorylation and formation of NFTs. Some FTDP-17 mutations cause tau pathology in neurons and glia cells

whereas in AD only neurons are affected (for an overview see Brandt et al., 2005). In nerve cells, hyperphosphorylated tau redistributes from the axon to the somatodendritic compartment where it aggregates into straight filaments (SFs) or paired helical filaments (PHFs) the main components of NFTs (Goedert et al., 1995). For AD it was shown that NFT formation occurs time- and regionspecific in the brain. First tau aggregates are found in the enthorhinal cortex, from where they spread to the hippocampus and the isocortex (Braak et al., 1993). Tau hyperphosphorylation may be caused by increased kinase or reduced phosphatase activity (for review see Blurton-Jones and Laferla, 2006). Although the number of NFTs correlates with the degree of cognitive decline in AD patients, whether aggregated or soluble tau is the main pathogenic tau species remains to be shown. Furthermore, the mechanism by which tau induces pathologic events is still unclear. Two hypothesises are currently discussed. The "loss of function" theory assumes that tau mutations or hyperphosphorylation or both cause a loss of the ability of tau for microtubule binding leading to microtubule destabilization, axonal transport disturbance and neuronal death. However, tau knock-out mice do not show major cytoskeletal abnormalities arguing against the suggested role of tau in axonal stability (Harada et al., 1994). Several studies reinforce as an alternative the "toxic gain of function" model which assumes that hyperphosphorylated tau, either soluble or in an aggregated state, has a direct toxic effect on neurons. Transgenic mice expressing the FTDP-17 tau mutant P301L showed NFTs and cell death (Lewis et al., 2000). Furthermore, expression of a hyperphosphorylation-mimicking tau construct in different culture systems caused massive neurodegeneration (Fath et al., 2002; Shahani et al., 2006; Tackenberg and Brandt, 2009), supporting a neurotoxic effect of disease-like modified tau.

2.3 Amyloid precursor protein (APP) and amyloid beta (Aβ)

Function and processing of the amyloid precursor protein (APP)

The amyloid precursor protein (APP) is a type I integral membrane protein and is expressed in many tissues. APP is the precursor of Aβ, which is the major component of amyloid plaques found in brains of AD patients. Aβ is supposed to be the key molecule in AD pathology. The production of Aβ occurs via cleavage of APP by different protein complexes, called secretases (Fig. 3). In the non-amyloidogenic pathway APP is cleaved by the α-secretase resulting in a membrane-bound C-terminal fragment (C83) and the soluble APPα (sAPPα)

which is thought to have neuroprotective properties (Stein et al., 2004). γ-secretase cleavage of C83 results in the formation of the APP intracellular domain (AICD) and the soluble P3 fragment. The AICD is involved in modulating gene expression (Müller et al., 2008), whereas the function of P3 is still unknown. In the amyloidogenic pathway β-secretase cleavage leads to the production of C99 and soluble APPβ (sAPPβ). Aβ is produced by a further cleavage of C99 by the γ-secretase. The γ-secretase is a membrane-embedded protein complex with presenilins (PS) as catalytic subunit. PS, which exists in two isoforms, PS1 and PS2, is also subjected to many FAD mutations. Other FAD mutations are found in the APP gene (Fig. 3, right). All these mutations facilitate the cleavage of either β- or γ-secretase and cause an increase in the amount of the most amyloidogenic form of Aβ, $Aβ_{42}$ (Steiner et al., 1999).

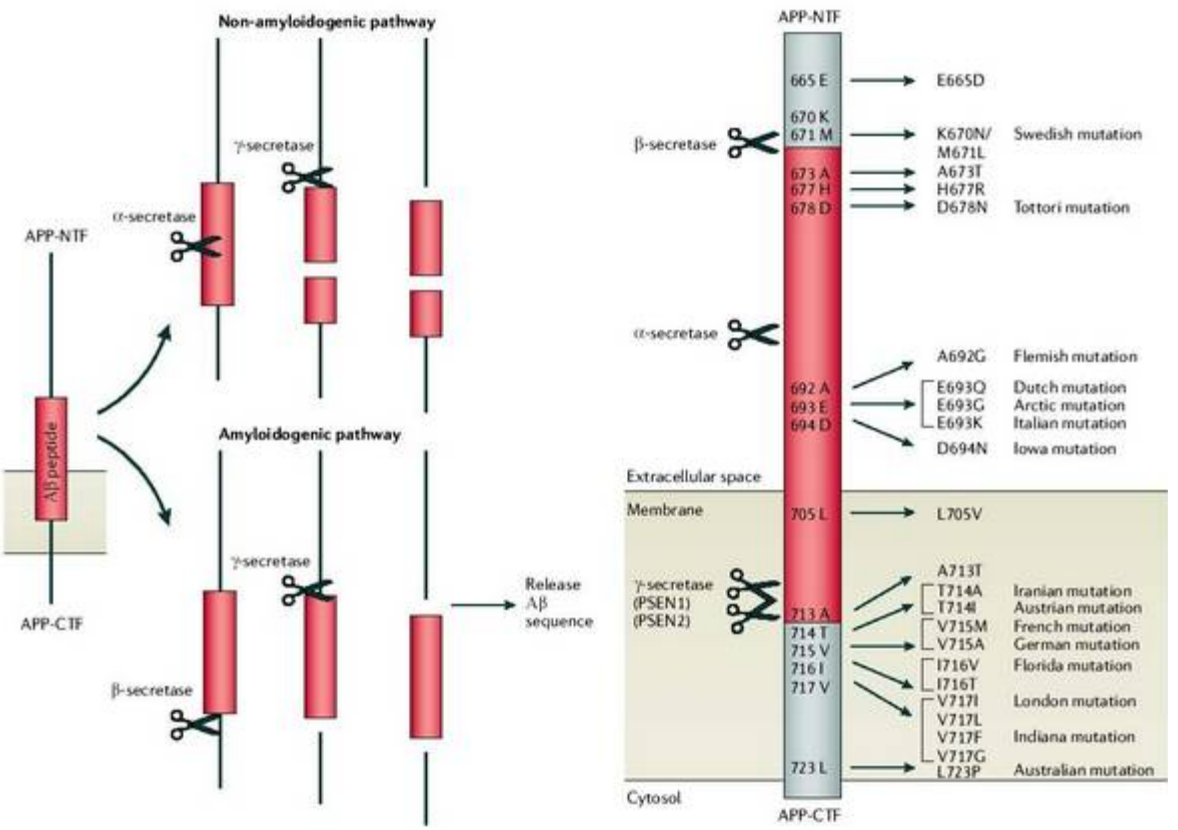

Fig. 3: Processing (left) and structure (right) of the amyloid precursor protein with sites of FAD mutations indicated for the longest APP isoform APP770 (Van Dam and De Deyn, 2006).

Several transgenic mice were created expressing FAD mutated APP. The mice used for this study express APP695 with three FAD mutations, the Swedish (KM595/596NL (sites for APP695 Isoform)), Dutch (E618Q) and London (V642I) mutation (Fig. 3 right). These mice produce equimolar amounts of $Aβ_{40}$ and $Aβ_{42}$ already in embryonic age. To elucidate the

physiological function of APP, which is still a matter of debate, APP knockout mice were constructed. These mice are viable and show besides impairment of spatial memory and long-term potentiation (LTP) only a weak phenotype (Ring et al., 2007). This may be explained by a complementation by the APP-like proteins (APLP1 and APLP2). A double knockout off APP and one of the APLPs or a triple knockout result in loss of viability (Wolfe and Guénette, 2007). Further, brain injury induces APP expression, which suggests that APP plays a repair role in this context. Other data provide evidence for a role of APP as membrane receptor similar to NOTCH (Wolfe and Guénette, 2007).

Amyloid beta peptide and the amyloid cascade hypothesis

The central role for the Aβ peptide in the pathologic processes in AD is widely accepted and is supported by the finding that FAD mutations increase Aβ production. One of the early events in AD is the alteration of the synaptical network in the brain as evidenced by loss of synapses and dendritic spines. Whether amyloid plaques or soluble Aβ species i.e. monomers, dimers or higher molecular weight oligomers are the main toxic Aβ species is still a matter of debate. Evidence for plaque-related alterations comes from Grutzendler et al (2007) who showed reduced synaptic contacts in the vicinity of amyloid plaques in PS1/APP double transgenic mice. However, it becomes more and more evident that soluble Aβ oligomers are the more toxic species. Various studies using cell cultures treated with soluble Aβ or APP transgenic mice showed strong effects of Aβ oligomers on synaptic complexity and on synaptic transmission (reviewed in Tackenberg et al., 2009). Recent studies found NMDA glutamate receptors (NMDARs) as a mediator of Aβ-induced changes (Shankar et al., 2007; Tackenberg and Brandt, 2009).

These and other studies support the amyloid cascade hypothesis of Alzheimer's disease attributing Aβ the central role as the initiator of the AD pathologic cascades being upstream of tau pathology (Hardy and Selkoe, 2002).

2.4 Interactions between Aβ and tau

According to the amyloid cascade hypothesis Aβ is upstream and induces tau pathology. Thus, the connection between extracellular Aβ and intracellular tau needs to be elucidated. As described above Aβ appears to be responsible for alterations of the synaptic network. However, some studies suggest that Aβ alone is not sufficient to induce cell death but that the presence of tau is essential. Rapoport et al (2002) reported that tau-expressing neuronal cultures strongly degenerated in the presence Aβ, whereas tau-depleted neurons did not. In another study using hippocampal slice cultures from APP transgenic mice massive neuronal loss was only observed in the presence of tau (Tackenberg and Brandt, 2009). As described above, tau is highly phosphorylated in AD, what may induce tau to become neurotoxic. Indeed, a hyperphosphorylation-mimicking tau mutant caused massive cell death in cell cultures (Fath et al., 2002; Shahani et al., 2006; Tackenberg and Brandt, 2009).
Increased expression or activition of several tau kinases is observed in AD, i.e. GSK-3β, Cdk5, Akt, MAPK, Abl, P59Fyn kinase and JNK. Several studies report that Aβ can induce tau hyperphosphorylation by activating these kinases. In APP transgenic mice increased JNK and MAPK activation was observed and a decrease in Aβ levels caused decreased activity of GSK-3β (reviewed in Blurton-Jones and Laferla, 2006). Additionally, downregulation of PP2A levels, one of the major tau phosphatases, is found in brains of AD patients (Vogelsberg-Ragaglia et al., 2001). Interestingly, tau hyperphosphorylation is also observed in FTDP-17 in the absence of Aβ, which may be caused by conformational changes due to the mutation in tau (reviewed in Brandt et al., 2005). However, no AD-related tau mutations are observed, confirming the role of Aβ as the main inducer of tau hyperphosphorylation in AD.

2.5 Sindbis virus expression system

A major challenge of current neuroscience is to understand the function of an enormous amount of proteins in the brain. To achieve this goal techniques are necessary to transfect postmitotic neurons with proteins of choice and monitor their effects. Today several methods are used to transfer genes into neurons including lipofection, ballistic transfer (“gene gun”) and viral infection. Viral vectors can be used to transfer DNA or RNA into dissociated cells, slice cultures and can also be applied *in vivo* (for an overview see Washbourne and McAllister, 2002). The Sindbis virus is a neurotropic RNA virus with very high infection

efficiencies (up to 95% of neurons) and high expression levels of the recombinant protein after few hours of infection (Ehrengruber et al., 2001). The infection and high protein expression levels cause cell damage after 1 day in dissociated cells and 3-5 days in slice cultures (Washbourne and McAllister, 2002), which requires careful controls for virus-induced toxicity. Since the Sindbis virus is closely related to Semliki Forest Virus (SFV) both are used as synonyms in the literature.

2.6 Organotypic *ex vivo* model of the hippocampus

The hippocampus formation is a brain region that belongs to the limbic system and plays an important role in long-term and spatial memory. It is named according to its shape which resembles a seahorse. It is one of the regions that are affected already early during the development of Alzheimer's disease.

Major hippocampal defects which may result from injury or disease cause a lack of the ability to form new memory, whereas existing memory remains, indicating that the hippocampus is not responsible for storage of memory but acts as a relay station.

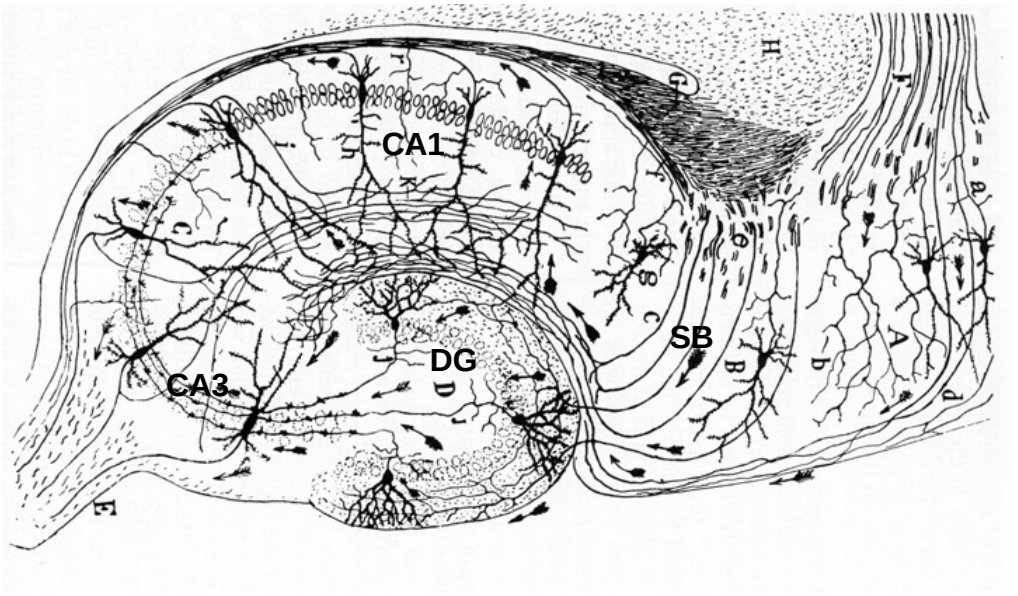

Fig. 4: Classsical drawing of the hippocampal architecture by Ramón y Cajal (Ramón y Cajal, 1911).

As seen in the drawings from Santiago Ramón y Cajal from 1911 (Fig. 4) the hippocampus has a one layer structure with the dentate gyrus (DG) forming the entrance. It obtains input

from the subiculum (SB) and other cortex regions via the perforant path. The dentate gyrus is characterized by granule neurons whose axons project as mossy fibres to the CA3 (cornu ammonis 3) region. This region contains large pyramidal neurons which form the Schaffer collaterals to form synaptic contact with CA1 neurons. These pyramidal cells have smaller call bodies than the CA3 neurons and their axons leave the hippocampus via the subiculum.
Hippocampal slices are used for diverse purposes as a well established method. Acute slice preparations are used for electrophysiological studies of LTP, a phenomenon of synaptic strengthening which is believed to play a major role in leaning and memory processes. Organotypic hippocampal slice cultures are useful to investigate pathophysiological changes in an authentic central nervous environment. Cells within these slices undergo differentiation and slices can be kept in culture for months (Duff et al., 2002). These cultures exhibit many *in vivo* aspects like the presence of functional synaptic networks and characteristic organotypic architecture thus can be considered as *ex vivo* models. They are easily accessible for experimental manipulation and allow the use of transgenic animals for their preparation. They are widely used to analyze synaptic complexity, neuronal survival and for drug screening purposes.

2.7 Dendritic spines

A characteristic feature of neurons is their polarity, separating the cell into a somatodendritic (signal receiving) and axonal (signal transducing) compartment. The dendrites of CA1 pyramidal neurons receive synaptic input from an average of about 30000 axons (Fiala and Harris, 1999). Pyramidal cells contain small dendritic protrusions which form a one to one contact to the presynaptic bouton. These structures are called dendritic spines and were first identified by Santiago Ramón y Cajal at the end of the 19th century (Fig. 5).

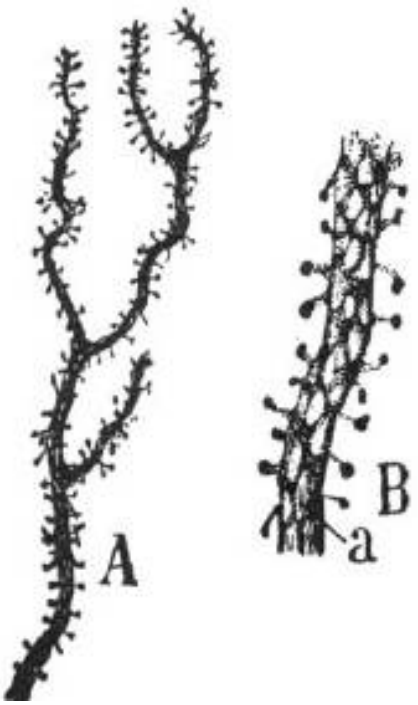

Fig. 5: Low (A) and high (B) magnification views of dendritic spines from a cerebellar Purkinje cell from a bird, drawn by Ramón y Cajal (Ramón y Cajal, 1899).

Ramón y Cajal already believed that these structures are involved in memory processes (Yuste, 2002). (See the first description of dendritic spines by Ramón y Cajal from 1888 in the Appendix). Decades later Donald Hebb postulated that the regulation of the strength of transmission between presynapse and spine could be the cellular correlate for learning and memory (Hebb, 1949). Since then, many studies confirmed that the interplay of synapse strengthening (LTP) and synapse weakening (long-term-depression, LTD) – together called synaptic plasticity - mediates learning and memory. LTP is induced by high frequent stimulation of a glutamatergic synapse leading to growth of the spine and insertion of more glutamate receptors making the synapse more stable, sensitive and functionally stronger. Although some cellular receptors and intracellular molecules responsible for synaptic plasticity have been identified, the signal transduction cascades and how they are altered in diseases such as AD remain to be shown (for a detailed overview see Tackenberg et al., 2009).

3. Results: Publications

Shahani N, Subramaniam S, Wolf T, Tackenberg C and Brandt R (2006)

Tau Aggregation and Progressive Neuronal Degeneration in the Absence of Changes in Spine Density and Morphology after Targeted Expression of Alzheimer's Disease-Relevant Tau Constructs in Organotypic Hippocampal Slices.

The Journal of Neuroscience 26:6103-14

Tackenberg C, Ghori A and Brandt R (2009)

Thin, stubby or mushroom: Spine pathology in Alzheimer's disease.

Current Alzheimer Research 6: 261-8

Tackenberg C and Brandt R (2009)

Divergent pathways mediate spine alterations and cell death induced by amyloid-beta, wt tau and R406W tau.

The Journal of Neuroscience, 29:14439-50

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The Journal of Neuroscience 26:6103-14

Abstract:

Alzheimer's disease (AD) is characterized by progressive loss of neurons in selected brain regions, extracellular accumulations of amyloid beta, and intracellular fibrils containing hyperphosphorylated tau. Tau mutations in familial tauopathies confirmed a central role of tau pathology; however, the role of tau alteration and the sequence of tau-dependent neurodegeneration in AD remain elusive. Using Sindbis virus-mediated expression of AD-relevant tau constructs in hippocampal slices, we show that disease-like tau modifications affect tau phosphorylation at selected sites, induce Alz50/MC1-reactive pathological tau conformation, cause accumulation of insoluble tau, and induce region-specific neurodegeneration. Live imaging demonstrates that tau-dependent degeneration is associated with the development of a "ballooned" phenotype, a distinct feature of cell death. Spine density and morphology is not altered as judged from algorithm-based evaluation of dendritic spines, suggesting that synaptic integrity is remarkably stable against tau-dependent degeneration. The data provide evidence that tau-induced cell death involves apoptotic as well as nonapoptotic mechanisms. Furthermore, they demonstrate that targeted expression of tau in hippocampal slices provides a novel model to analyze tau modification and spatiotemporal dynamics of tau-dependent neurodegeneration in an authentic CNS environment.

Tackenberg C, Ghori A and Brandt R (2009)

Thin, stubby or mushroom: Spine pathology in Alzheimer's disease.

Current Alzheimer Research 6: 261-8

Abstract:

Since their first description by Ramon y Cajal at the end of the 19th century, dendritic spines have been proposed as important sites of neuronal contacts and it has been suggested that changes in the activity of neurons directly affect spine morphology. In fact, since then it has been shown that about 90% of excitatory synapses end on spines. Recent data indicate that spines are highly dynamic structures and that spine shape correlates with the strength of synaptic transmission. Furthermore, several mental disorders including Alzheimer's disease (AD) are associated with spine pathology suggesting that spine alterations play a central role in mental deficits. The aim of this review is to provide an overview about the current knowledge on spine morphology and function as well as about different experimental models to analyze spine changes and dynamics. The second part concentrates on disease-relevant factors that are associated with AD and which lead to spine alterations. In particular, data that provide evidence that Abeta oligomers or fibrillar Abeta deposits influence spine morphology and function will be presented and the contribution of tau pathology will be discussed. The review ends with the discussion of potential mechanisms how disease-relevant factors influence dendritic spines and whether and how spine changes could be therapeutically suppressed or reversed.

Tackenberg C and Brandt R (2009)

Divergent pathways mediate spine alterations and cell death induced by amyloid-beta, wt tau and R406W tau.

The Journal of Neuroscience, 29:14439-50

Abstract:

Alzheimer's disease is characterized by synaptic alterations and neurodegeneration. Histopathological hallmarks represent amyloid plaques composed of amyloid-β (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau. To determine whether synaptic changes and neurodegeneration share common pathways, we established an *ex vivo* model using organotypic hippocampal slice cultures from amyloid precursor protein transgenic mice combined with virus-mediated expression of EGFP-tagged tau constructs. Confocal high-resolution imaging, algorithm-based evaluation of spines, and live imaging were used to determine spine changes and neurodegeneration. We report that Aβ but not tau induces spine loss and shifts spine shape from mushroom to stubby through a mechanism involving NMDA receptor (NMDAR), calcineurin, and GSK-3β activation. In contrast, Aβ alone does not cause neurodegeneration but induces toxicity through phosphorylation of wild-type (wt) tau in an NMDAR-dependent pathway. We show that GSK-3β levels are elevated in APP transgenic cultures and that inhibiting GSK-3β activity or use of phosphorylation-blocking tau mutations prevented Aβ-induced toxicity of tau. FTDP-17 tau mutants are differentially affected by Aβ. While R406W tau shows increased toxicity in the presence of Aβ, no change is observed with P301L tau. While blocking NMDAR activity abolishes toxicity of both wt and R406W tau, the inhibition of GSK-3β only protects against toxicity of wt tau but not of R406W tau induced by Aβ. Tau aggregation does not correlate with toxicity. We propose that Aβ-induced spine pathology and tau-dependent neurodegeneration are mediated by divergent pathways downstream of NMDAR activation and suggest that Aβ affects wt and R406W tau toxicity by different pathways downstream of NMDAR activity.

4. Conclusions and Outlook

Features of AD are massive cell death in selected brain regions and loss of presynaptic boutons and postsynaptic spines. Histopathological hallmarks are Aβ plaques and aggregates of hyperphosphorylated tau protein. Whether or how Aβ and tau contribute to cell death and synapse loss in AD remains to be elucidated. To analyze a potential role of tau in mediating neuronal death and spine loss, an *ex vivo* system was established. Organotypic hippocampal slice cultures were prepared from mice and infected with Sindbis virus to express EGFP-coupled wt tau and hyperphosphorylation-mimicking PHP tau in neurons, the latter to simulate a disease-like tau modification (Shahani et al., 2006). It could be shown that overexpression of wt tau does not induce cell death or alter density or morphology of dendritic spines. In contrast, PHP tau induced massive neurodegeneration. Interestingly, dendritic spine number and morphology were not affected. Thus, tau hyperphosphorylation may trigger neuronal degeneration during AD whereas the decrease in synaptic density may not be caused by tau alone but by a mechanism requiring the presence of Aβ.

To analyze the role of Aβ and functional interactions of tau with Aβ, *ex vivo* cultures were prepared from APP_{SDL} transgenic mice and non transgenic littermates (Tackenberg and Brandt, in press). APP_{SDL} transgenic mice express human APP695 with a combination of three familial Alzheimer's disease mutations, the Swedish (KM595/596NL), Dutch (E618Q) and London (V642I) mutation (Blanchard et al., 2003). These mutations cause the production of equimolar amounts of $Aβ_{40}$ and $Aβ_{42}$ already in embryonic age (Leschik et al., 2007). The slices were infected with Sindbis virus expressing EGFP or EGFP-coupled tau constructs. High resolution imaging after expression of EGFP or EGFP-coupled wt tau showed a drastic reduction in density and length of dendritic spines and a shift in spine shape from mushroom to stubby in APP transgenic slices compared to non transgenic controls. In contrast, low resolution live imaging of infected neurons in APP transgenic slices showed that the presence of the transgene alone does not cause neuronal death but requires the presence of wt tau to induce neurodegeneration. Western blot analysis using phosphorylation sensitive tau antibodies showed increased wt tau phosphorylation at PHF-1 site in APP transgenic slices compared to non transgenic controls, suggesting that Aβ induces tau toxicity by increased phosphorylation. In agreement, phosphorylation blocking mutations prevented Aβ-induced tau toxicity. The glycogen-synthase-kinase-3β (GSK-3β) is one of the major tau kinases.

GSK-3β levels were elevated in APP transgenic cultures making it possible that Aβ-induced tau phosphorylation was mediated by GSK-3β.

Since many mouse models combine FAD mutated APP or PS1 with FTDP-17 mutated tau the interaction of Aβ with two FTDP-17 tau mutants (R406W and P301L tau) was analyzed. Expression of R406W tau caused slight cell death in non transgenic slices and massive degeneration in APP transgenic cultures. In contrast, expression of P301l tau was not toxic neither in the absence nor in the presence of Aβ.

To determine whether the different behaviour of wt, R406W and P301L tau was due to different solubility profiles, sequential tau extraction was performed using buffers of increasing stringency. No correlation between tau aggregation and toxicity was found.

The *ex vivo* approach also permits to analyze signal transduction pathways involved in spine changes and neurodegeneration. Blocking Aβ production with DAPT abolished both spine changes and neurodegeneration. This suggests that Aβ is upstream of tau and confirms the amyloid cascade hypothesis of AD. NMDAR activity was required for both induction of spine alterations and Aβ-induced toxicity of wt tau and R406W tau. GSK-3β activity was essential for both pathologic processes whereas calcineurin was only involved in mediating spine changes. Interestingly, the induction of R406W tau toxicity by Aβ was GSK-3β independent. In agreement, R406W tau phosphorylation at PHF-1 site was not increased by Aβ, indicating that Aβ affects wt tau and R406W tau by different mechanisms downstream of NMDAR activation.

For future work it would be interesting to determine the functional consequences of morphological spine changes caused by Aβ by electrophysiological measurements. Furthermore, it could be analyzed if the morphological spine changes also occur *in vivo* in APP_{SDL} transgenic mice at different ages. Currently, our light microscope does not support the generation of z-stack images from golgi-stained brain slices for analysis with 3DMA-Neuron software.

The signal transduction cascades and mechanisms causing spine pathology and cell death could be analyzed in more detail. Many potential effectors of both processes are under investigation, including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), Cofilin and several tau kinases (reviewed in Blurton-Jones and Laferla, 2006 and Knobloch and Mansuy, 2008). This could provide the possibility to determine potential drug targets for disease-modifying therapies.

The different behaviour of wt tau and R406W tau in the absence and presence of Aβ could be elucidated to determine the mechanism of tau-induced cell death in FTDP-17 cases where no amyloid pathology occurs. It may also be useful to combine the ten phosphorylation-blocking mutations in Ala tau with the R406W tau mutation to analyze whether a phosphorylation at these sites causes toxicity of R406W tau in the presence or absence of Aβ.

Additionally, the absence of toxicity of P301L tau in our model is remarkably since this mutation strongly induces neurodegeneration in patients suffering from FTDP-17. In a study from Andorfer et al (2003) it could be shown that endogenously expressed mouse tau protected against toxicity human tau in transgenic mice, whereas expression of human tau induced AD-like tauopathy in the absence of mouse tau. Thus, endogenous mouse tau may have neuroprotective activity. To determine the effect of endogenous mouse tau in our culture system, our mice could be crossed with $tau^{-/-}$ mice. This may allow toxicity of P301L tau in case it is masked by endogenous mouse tau. It would also be interesting to determine whether Aβ itself, which was not toxic in the presence of endogenous tau, may induce cell death in the absence of endogenous tau.

Although our data point to the central role of Aβ in the pathologic processes it remains to be shown which type of Aβ triggers pathology. While a 56 kDa Aβ peptide (Abeta*56) was identified to disrupt synaptic contacts in APP transgenic mice in early stages (Lesné et al., 2006), Shankar et al (2008) revealed smaller Aβ dimers as the most synaptotoxic Aβ species. Thus, it will be interesting to determine which Aβ species are present in our model.

Several studies have focused on the impact of Aβ on the actin cytoskeleton in spines by induction of LTD (reviewed in Tackenberg et al., 2009). However, it could also be shown that LTP induces the formation of microtubule tracks from the soma into the spine which may be the tracks for AMPA receptor trafficking to the spine (Mitsuyama et al., 2008). Thus, it could be interesting to determine whether this mechanism may also be affected by Aβ or by hyperphosphorylated tau protein.

The development of drugs to inhibit Aβ production and aggregation or to increase Aβ clearance has become a central part of research in this field. Furthermore, drugs to inhibit tau phosphorylation e.g. kinase inhibitors are under investigation. Our data indicate that the new *ex vivo* system provides a valid model which could be used for drug screening since it resembles many *in vivo* aspects while being much better accessible for manipulation.

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6. Appendix (I)

Interviews of Auguste D. by Alois Alzheimer (Dahm, 2006)

Questions of Alois Alzheimer are written in italic.

Nov 26th, 1901:

What is your name?

Auguste.

Surname?

Auguste.

What is your husband's name?

I believe Auguste.

Your husband?

I see, my husband… [She looks as if not having understood the question.]

Are you married? Mrs. D.?

Yes, to Auguste D.

How long have you been here?

[She appears to be trying to remember.] Three weeks.

At lunch she eats pork and cauliflower. When asked what she is eating, she replies spinach. When chewing meat and asked what it was, she answers potatoes and then horseradish.

Nov 29th, 1901:

How are you?

It is always one as the other. Who carried me here?

Where are you?

At the moment, I have temporarily, as I said, I have no means. One simply has to… I don't know myself… I really don't know… dear me, what is to then?

What is your name?

Frau Auguste D.

When were you born?

Eighteen hundred and…

In which year were you born?

This year, no, last year.

When were you born?

Eighteen hundred — I don't know.

What did I ask you?

Ah, D. Auguste. […]

What is your husband called?

I don't know.

What is your husband's name?

[She answers quickly and as if suddenly awoken.] August Wilhelm Karl; I don't know if I can state it like this.

What is your husband's profession?

Clerk — I am so confused, so confused, I can not. […]

What year is it?

Eighteen hundred…

Which month?

The 2nd. […]

If you buy 6 eggs, at 7 pfennig each, how much is it?

To poach.

What street do you live on here?

I can tell you, I just have to wait a bit.

What did I ask you?

Well, this is Frankfurt am Main.

On what street do you live?

I can tell you, Waldemarstraße, not, no…

When did you marry?

I don't know at present. The woman lives on the same floor.

Which woman?

The woman where we are living. [The patient calls out] Mrs Hensler, Mrs Hensler… here one step below, she lives. [Alzheimer shows her different objects; she names them correctly.]

What did I show you?

I don't know… I don't know… so anxious, so anxious.

How many fingers? [Alzheimer shows her three fingers.]

Three.

Are you still anxious?

Yes.

How many fingers did I show you?

Well, it is Frankfurt am Main…

Appendix (II)

First description of dendritic spines by Santiago Ramon y Cajal in 1888:

"Also, the surface of the Purkinje cells' dendrites appears bristling with thorns or short spines, which in the terminal branches are represented by light asperities. Early on we thought that these eminences were the result of a tumultuous precipitation of the silver; but the constancy of their existence and its presence even in preparations where the reaction appears with great delicacy in the remaining elements, incline us to consider them as a normal disposition"
(cited by Yuste, 2002)

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Last but not least I want to thank my wife Maria for her extraordinary support.

Thank you.

8. Curriculum Vitae

Name: Christian Tackenberg

Date, place of birth, family status

- October 29th 1978, Osnabrück (Germany), married, two children

Education

- 1985-1998 Elementary school, orientation stage, grammar school
- 1998 High school exam at the Graf-Stauffenberg-Gymnasium in Osnabrück

Scientific training

- 1999-2005 Studies of Biology at the Universities of Osnabrück and Hohenheim
- 2005 Diploma in Biology ("very good") at the University of Hohenheim, Institute for Micro- and Molecular Biology, Prof. Dr. Andreas Kuhn; Title: "Aq-175: Das YidC-Homolog aus *Aquifex aeolicus* – Klonierung und heterologe Expression in *Escherichia coli"*
- 2005-2008 associated member of the graduate college 612 at the University of Osnabrück
- Since 2005 Doctoral thesis in the Department of Neurobiology at the University of Osnabrück with the title: "Effect of amyloid precursor protein and tau on dendritic spines and cell survival in an *ex vivo* model of Alzheimer's disease

Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.
Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich / unentgeltlich geholfen.

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Weitere Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
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