



Review

Alzheimer's disease: Old problem, new views from transgenic and viral models

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ABSTRACT

Alzheimer's dementia is developing ever more as a complex syndrome with various unknown genetic and epigenetic contributions. These are compounded on and exacerbating the underlying amyloid and tau pathology that remain the basis of the pathological definition of Alzheimer's disease. Here, we present a selection of aspects of recent bigenic and virus-based mouse strains, developed as pre-clinical models for Alzheimer's disease. We discuss newer features in the context of the characteristics defined in previously validated transgenic models. We focus on specific aspects of single and multiple transgenic mouse models for Alzheimer's disease and for tauopathies, rather than providing an exhaustive list of all available models. We concentrate on the content of information related to neurodegeneration and disease mechanisms. We pay attention to aspects and defects that are predicted by the models and can be tested in humans. We discuss implications that help translate the fundamental knowledge into clinical, diagnostic and therapeutic applications. We elaborate on the increasing knowledge extracted from transgenic models and from newer adeno-associated viral models. We advocate this combination as a valuable strategy to study molecular, cellular and system-related pathogenic mechanisms in AD and tauopathies. We believe that innovative animal models remain needed to critically test current views, to identify and validate therapeutic targets, to allow testing of compounds, to help understand and eventually treat tauopathies, including Alzheimer's disease.

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1. Introduction and background

1.1. Alzheimer disease

Alzheimer's disease (AD) is the most prevalent form of dementia among the elderly accounting for more than 70% of all dementia cases [1]. The clinical disease stages are initially announced by mild cognitive impairment (MCI) that gradually progresses from subtle cognitive and memory problems to severe deficits, inevitably ending in deep dementia. The composite clinical picture at any stage is variable among AD patients, including cognitive and memory problems, and mild to severe changes in social behavior with mood swings and altered personality and character, including apathy, depression, irritability, agitation, psychosis, aggression.

Current treatments for AD are purely symptomatic and hardly effective. The development of disease-modifying therapies is extremely urgent because of the exponential increase in AD with age. This poses a direct and major medical and social threat for current and all future generations with our ever-increasing life expectancy.

Diagnosis of AD is not uniformly accepted or applied, and based on variable combinations of neurological examination, CSF-biomarkers, mental and memory tests, MRI and PET brain-imaging. Needless to state that, even when effective therapy becomes available, diagnosis of AD should be made as early as possible in the disease process to help the patient, the family and caretakers.

The development of efficient diagnostic and therapeutic means relies entirely on scientific progress made in understanding the fundamental mechanisms that cause and underlie AD. Progress then remains heavily dependent on studies in animal models that recapitulate the disease at least in essentials aspects, if not as exact and complete phenocopies.

Moreover and in addition, early objective diagnosis of AD is essential for many reasons. Today it is profoundly hampered, if not made impossible by problems of accuracy and safety, specificity and reproducibility of current methods and tests, ranging from cognitive examination tests, lumbar puncture and ELISA for CSF-biomarkers, PET-imaging for amyloid load and glucose metabolism, MRI for brain region-specific atrophy. Many tests detect problems (too) late in the disease process to allow effective treatment, provided it was available. Diagnostic problems therefore reverberate into therapeutic tests, because of difficulties with recruiting properly diagnosed AD patients, and of proper stratification by genetic, clinical or biochemical parameters. This is currently technically impossible and prevents

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personalized therapeutic strategies that would be more effective and less costly. The same problems plague experimental therapies that are hampered by the objective estimation of the rate of decline of cognition. Finally, stratification based on genetic parameters remain largely limited to defining the ApoE genotype, and is used often only post-hoc. The many genetic, epigenetic and environmental factors that are claimed as potential risk-factors to AD are intensely debated, but none generally accepted or implemented as objective parameters in clinical studies – with exceptions on the obvious parameter of age and gender.

Despite all the technical progress, the post-mortem brain pathology described by Alzheimer more than a century ago, still defines AD. The microscopic pathology remains the only final diagnosis, unfortunately post-mortem: extracellular amyloid plaques made up of aggregated amyloid peptides and intracellular neurofibrillary tauopathy resulting from aggregated phosphorylated protein tau. The associated parameter of inflammation, also noted by Alzheimer, remains the third important hallmark in AD brain.

1.2. AD and amyloid

A panoply of data corroborates the amyloid cascade hypothesis that proposes a primary, causal role for amyloid peptides in the pathogenesis of AD. The experimental proof is, however, still not conclusive despite 25 years of molecular and cellular analysis since the discovery of amyloid peptides [2]. Amyloid peptides (A β) accumulate in the brain of AD patients because of increased production or of impaired elimination. The peptides are produced from the amyloid precursor protein (APP) by a complex set of sequential endoproteolytic cleavages [3].

Amyloidogenic processing of APP is initiated by β -secretase (BACE) that produces the secreted APPs β ectodomain and the cell-bound C99 fragment, which is then cleaved by γ -secretase to release the intracellular domain (AICD). This process also produces various amyloid peptides for as yet unknown biological reasons, because no physiological function is unequivocally assigned to any of the amyloid peptides.

Non-amyloidogenic processing of APP results in the secreted APPs α ectodomain and the C83 fragment, following cleavage by α -secretase, an activity exerted mainly by ADAM10 [4]. The C83 fragments are also processed by γ -secretase to yield the same AICD fragment, but also the harmless p3 peptides instead of the amyloid peptides [3].

Disease-modifying therapies in clinical tests today are mostly aimed at reducing the amyloid peptide concentrations in brain, either (i) by inhibition of β - or γ -secretases, (ii) by increasing elimination by active or passive immunization, (iii) by preventing peptide aggregation, or (iv) by increasing proteolytic degradation. Despite the many drawbacks and problems in these different approaches, the amyloid peptides remain prime target for therapy, and prime suspects for the pathogenic mechanism in AD [5].

Interestingly, PET-imaging using specific amyloid-ligands demonstrated high amyloid-load in the brain of cognitive non-compromised elderly and concluded to 20–40% false amyloid-positive cases pending the study [6–8]. These findings imply that even brain amyloid load does not equate to impaired cognition or failing mental capabilities.

1.3. AD and tau: not an innocent by-stander

In contrast to many primary tauopathies, wherein tau is accountable for neurodegeneration and its consequences, AD is classified as a secondary tauopathy downstream of amyloid pathology. Protein tau is yet to be generally accepted as being important in the overall disease process.

Tauopathy is typified by neurofibrillary tangles and neuropil treads composed of phosphorylated protein Tau and constitutes the second post-mortem diagnostic hallmark in AD but in contrast to amyloid pathology, is not specific for AD. Tauopathy is diagnostic for a

variety of neuro-degenerative diseases that differ widely clinically, biochemically and pathologically [9–11].

Importantly, tauopathy is invariably present in all AD cases, including the early onset familial cases (EOFAD) that are associated with dominant mutations in the genes coding for APP or presenilins. These presenile AD-cases are by definition caused by excess production of deviating amyloid peptides. Consequently, the associated tauopathy in these obligate amyloid AD cases, must be closely linked to the cognitive demise and dementia of the Alzheimer type. Arguably, this is the strongest argument for an essential pathological contribution of the tauopathy in AD, rather than an innocent bystander phenomenon. In addition, long-standing observations maintain that the typical brain-regional occurrence and progression of tau pathology in AD patients correlates temporally and spatially more closely with neuronal and cognitive dysfunction than amyloid pathology in AD [12,13].

Interestingly, no familial “amyloid-only” AD-cases have been described, while more than 40 mutations in the gene coding for human protein tau (the MAPT gene on chromosome 17) are associated with many sub-types of frontotemporal dementia [14,15] (cfr www.alzforum.org). These mutations are either exonic and expressed as a mutant protein tau, or intronic, which affect splicing of the tau mRNA to include or exclude exon10 that codes for the second microtubule binding domain. Importantly, intronic mutations produce normal protein Tau, but at deviating levels, which disturbs physiological functions that lead to synaptic and neuronal failure and degeneration.

1.4. Protein Tau, microtubule binding and tauopathy

While the amyloid cascade hypothesis [5] emphasizes the importance of amyloid peptides to the underlying pathology in AD, it does not provide an explanation for the inherent tauopathy associated with all AD cases. In addition, as discussed in the previous section, transgenic mouse with amyloid pathology caused by neuronal expression of any APP, wild-type or mutant, do not develop authentic tauopathy.

The aggregation of phosphorylated protein Tau into filamentous inclusions, eventually tangles, is the characteristic pathological feature of many neurodegenerative disorders known as Tauopathies, including Pick's disease, progressive supranuclear palsy, frontotemporal dementia (FTD) and many others [10,11,16–18]. Familial forms of tauopathy are associated with exonic and intronic mutations in the MAPT gene coding for protein tau on chromosome 17 in humans. The mutations are linked to various subtypes of fronto-temporal dementia, typified by extensive tau pathology in frontal and temporal brain regions, largely without any other associated pathology, i.e. no amyloid deposits.

The two most prevalent isoforms of tau, Tau3R and Tau4R, originate by alternative splicing of exon10 and have 3 or 4 microtubule binding repeats [19]. Tauopathies are differentiated biochemically by the ratio of Tau4R/Tau3R as well as by the relative composition of all tau-isoforms in the aggregates or tangles. In the case of familial FTD, the associated mutations are the defining factor, e.g. P301L, G272V, N279K, V337M, R406W, among others. To understand the mechanisms causing the familial forms of tauopathy linked to the MAPT gene, we must define an aberration in protein tau that is both needed and sufficient to cause neurodegeneration – in the absence of amyloid. The eventual understanding of the problem is even more concerned by the fact that many intronic mutations in specified FTD-families are located in intron-exon splice sites flanking exon10, encoding the second microtubule binding domain in protein tau. These intronic mutations evidently produce normal wild-type protein Tau, and their contribution to FTD can only be a distorted ratio of tau-isoforms in CNS.

The only known physiological action of protein tau is binding to microtubules, and its affinity is proportional to the number of microtubule binding domains: Tau4R binds stronger than Tau3R. Tau3R predominates in embryonic and fetal development, which is thought to allow greater plasticity and remodeling of the cytoskeleton of

neurons in the developing CNS. In adult human brain, Tau4R isoforms are more evident over Tau3R, while adult mouse brain expresses only Tau4R. Whether the human–mouse difference contributes to the lack of tauopathy in APP mice remains open for debate.

The expressed exonic mutations resulting in a mutant Tau4R protein that are associated with familial FTD, have been claimed to reduce the affinity of binding to microtubuli and/or to decrease microtubule assembly promotion.

Interestingly, the tau-microtubule physiological interaction is proposed to involve formation of tau homo-dimers [20]. This theoretical model accounts convincingly for the observed trans-sectional spacing between microtubules in axons, needed to allow transport of bulky cargo by the motor proteins. The binding of protein tau to microtubuli is dynamically regulated by a complex pattern of phosphorylation by different kinases. This review does not focus on this aspect, although it is of prime importance for the normal physiological function of protein tau, and for its deviation in tau pathology that is invariably characterized by excessive tau-phosphorylation [21–25]. The challenge is to define among the more than 10^{11} possible phosphorylated tau combinations those that tilt protein tau from MT-bound into semi-crystalline fibrillar polymer. It must be remembered that protein tau is an extremely soluble, naturally unfolded protein without a well-defined 3-dimensional structure that withstands boiling and precipitation by chaotropic agents. But these protein-structural characteristics do not explain the enigmatic processes that govern its aggregation in the cytoplasm of neurons in the various tauopathies.

These and other features raise even more questions on brain-regional differences of tauopathies with their wide variation in molecular, physical and pathological aspects and with tau-aggregates that contain either Tau3R or Tau4R isoforms, or mixtures. Moreover, while we can understand that phosphorylated tau appears in the CSF of patients as a result of neuronal damage or cell-death, it remains totally unclear why protein tau is present in CSF of cognitive normal individuals. These and more questions are to be addressed and answered *in vivo*, in suitable animal models.

2. Hypothesis: amyloid, but not alone

The combined evidence positions amyloid peptides as the initiators or trigger in AD, likely as oligomers and/or small aggregates that are not exactly defined molecularly and physically.

Protein tau is more and more proposed as a central actor or even executor of synapses and neurons in AD, largely by extrapolation from primary tauopathies.

How protein tau exerts its normal physiological function as microtubule-associated protein and how and why in other conditions, mutations or changed levels force protein tau to adopt a pathological role, remains largely to be discovered *in vivo*.

3. *In vivo* pre-clinical models

3.1. Problems and solutions, questions and answers

The disappointing outcome of clinical studies aimed to decrease, curb or neutralize amyloid peptides or their effects, increases the awareness that effective treatment of AD will not be obtained by mono-therapy. The AD-problem is vast and variable, and will require multi-targeted combinational approach, which next to amyloid proposes tauopathy as obvious target.

To make progress that is physiologically sound, pathologically relevant and therapeutically effective, we must know physiological functions and pathological roles of APP and its complex proteolytic processing. The same holds for protein tau and its intricate web of kinases and modifying enzymes causing the extensive post-translational modifications, not only phosphorylations but also proteolytic truncation, O-glycosylation, glycation, ubiquitinylation, ... Physiolog-

ical and pathological contributions are to be defined in the context of the functions of tau, its interacting partners, its dynamic control and its turnover. This vast task can be elucidated in appropriate validated models, and here we concentrate on transgenic mice.

The recapitulation of the typical combined AD-pathology *in vivo* closely followed knowledge of molecular mechanisms. The major challenge is still actual after 20 years of generating transgenic mouse models that have been invaluable for understanding molecular details central to AD. Non-mammalian models have been generated to study cellular pathways and protein interactions that can contribute to the pathogenesis. Transgenic flies, nematodes, zebrafish, lamprey, yeast and mammalian cells, among others have been generated as models for tau-induced neuro-degeneration, with limited success. These models are “less complex” than mammals and have offered valuable leads into the problems at hand, but they do not match the mammalian brain that is accessible in transgenic mice. The question remains pertinent to what extent we can hope to recapitulate the complexity of neuropathological, cognitive and behavioral features of AD in a small, short-lived mammal. The question implies whether we should continue to recapitulate AD in a single mouse model, no matter how many genes or mutations we have to include or should we study partim-aspects of AD in incomplete models *in vivo* [26].

Our research-group generated and characterized over the last two decades many different single and multiple transgenic mouse strains, resulting in validated models for amyloid and tau pathology [27–31]. Recently we produced and characterized bigenic APP.V717I \times Tau.P301L mice (biAT) as more complete model with synergistic amyloid and tau pathology in limbic brain regions [32]. The parallel development of the biGT tauopathy model by combining Tau.P301L and GSK3 β mice defined the essential role of GSK3 β in tauopathy [32,33].

We have now extended our armamentarium by including a virus-based model, using the hybrid adeno-associated viral vector (AAV1/2) to elicit more rapidly than in transgenic models aspects of amyloid and tau metabolism *in vivo* in the hippocampus of wild-type mice [34]. The outcome was rather surprising as these AAV-models define a major role for protein tau in neurodegeneration.

Here we present and discuss selected aspects of these models in the context of previously developed and validated transgenic models for amyloid and tau pathology. We focus this review on specific aspects of single and multiple transgenic mice for AD and for tauopathy alike, rather than drafting an exhaustive list of available models. We concentrate on lessons that we have learned from the mouse models and discuss the implications for translating fundamental knowledge into clinical, diagnostic and therapeutic applications. We are convinced that the combination of transgenic with viral models will contribute to our understanding of molecular, cellular and system-related pathogenic mechanisms. The models will contribute to identify therapeutic targets, and allow testing compounds to eventually treat primary and secondary tauopathies, including AD.

3.2. Older models, older views: mutant APP mice recapitulate amyloid pathology

Most efforts to generate mouse models for AD were based on the expression of mutant APP genetically linked to familial early-onset AD (EOFAD) using various gene promoters in different mouse genetic backgrounds. Innumerable efforts resulted in many models with different spatial and temporal expression patterns and even more important variations in expression levels. Here we discuss these models only briefly because comprehensive reviews are available [27,35–39]. An extensive, albeit not complete list of transgenic models for AD can be consulted on-line (www.alzforum.org).

The wanted characteristic of proficient APP transgenic mouse strains is robust and predictable amyloid pathology, comparable to amyloid pathology in AD patients. Amyloid pathology was and still is considered widely as the most essential feature of an AD-model. The time-line of its

appearance begins early, in some models only weeks after birth, with intracellular amyloid accumulation in various types of vesicles (Fig. 1). APP-mice then develop diffuse extracellular deposits and finally dense amyloid plaques, usually at an age that exceeds 1 year. Plaques stain with congo-red, thioflavinS and similar compounds with high affinity for extensive β -pleated sheets of aggregated proteins, similar to AD brain. Dense focal amyloid plaques are surrounded by dystrophic neurites and associated with activated glia, typical for inflammatory processes, again as in AD brain. In many models, vascular amyloid deposits develop subsequent to parenchymal plaques, resulting in congophilic amyloid angiopathy (CAA) typical for AD brain [29,40,41, and references therein]. Interestingly, we observed that microbleeds are dependent on the genetic background of the mice, which is relevant for the situation in AD patients. Indeed, the incidence of microbleeds and small vascular accidents varies in different studies. It is evident in no more than 20–35% of all patients, likely reflecting genetic predilection in individuals at risk for vascular problems.

The amyloid load onset and extent in APP models appears to be determined most importantly by the level of expression of the APP-transgene and by the type and number of dominant mutations incorporated, i.e. up to 5 in some models. Thereby, the relative concentration of fibrillogenic amyloid peptides in the brain, mainly

indexed as the ratio $A\beta_{42}/A\beta_{40}$, appears defining for the ensuing amyloid pathology. Data combined from single and bigenic mice, incorporating mutant PS1, assigns a major contributions in amyloidogenesis to $A\beta_{42}$ peptide as seed and to both $A\beta_{42}/40$ as bulk material for amyloid plaques in parenchyma. We consider the combination of mutant APP with mutant PS1 not the most valuable model for amyloid pathology, nor for its repercussions, because the mechanisms imposed differ considerably from ageing, which remains the major risk-factor for sporadic AD [42].

Remarkably, neuronal expression of mutant APP is sufficient to cause not only plaques in the parenchyma, but also vascular amyloid deposition. This supports the hypothesis that amyloid peptides produced by neurons diffuse in the interstitial fluid and are eventually drained via perivascular spaces to the blood or cerebrospinal fluid [29,43,44, and references therein]. The dynamic relation of parenchymal to vascular amyloid deposits was convincingly corroborated in bigenic APP.V717I x BACE mice, which produce more of shorter $A\beta_{11-40/42}$ peptides truncated by excess BACE activity. The N-truncated amyloid peptides evidently are less soluble and dramatically increased the parenchymal plaque-load while shunting vascular amyloid [45]. The data corroborate the hypothesis that temporal and spatial amyloid deposition in brain parenchyma and vasculature is determined by total

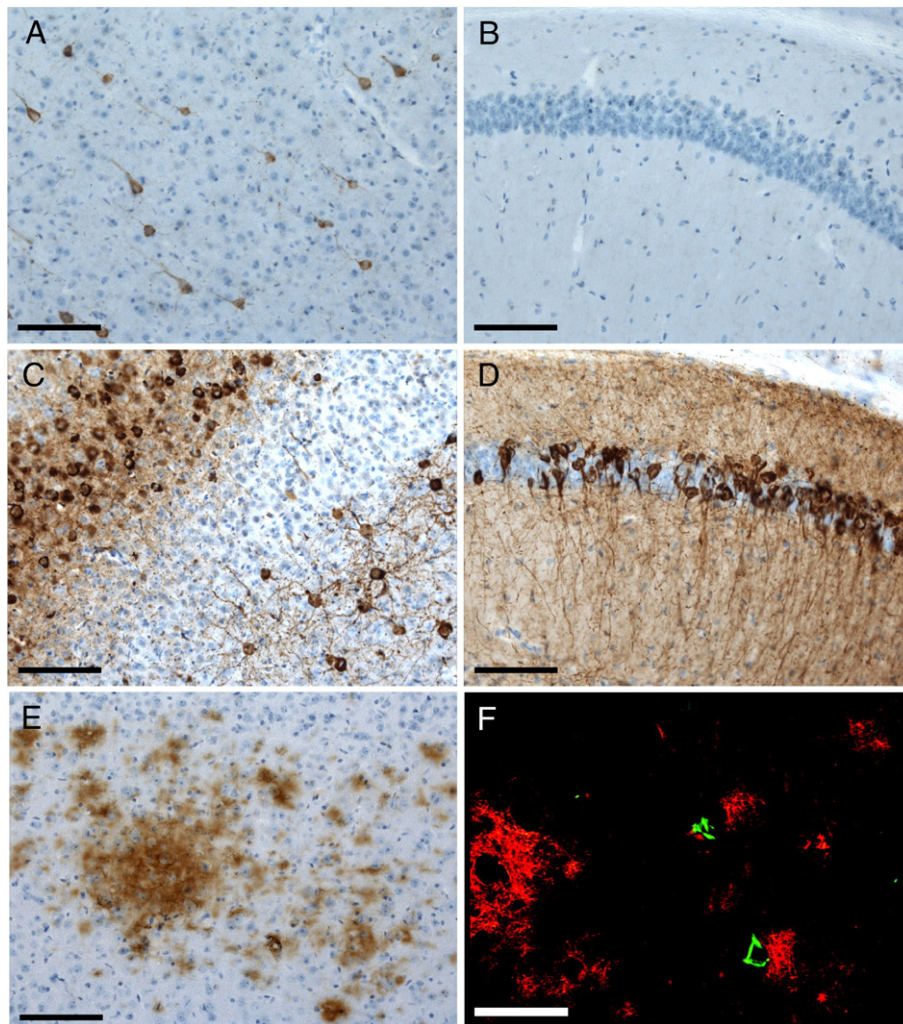


Fig. 1. Amyloid pathology and tauopathy in brain of transgenic and bigenic mice. Immunohistochemical staining on floating sections with specific monoclonal antibodies, as described in studies referred to. (A, B) phospho-Tau with AT100 in frontal cortex (A) and hippocampus (B) of terminal Tau.P301L mouse [57]. (C, D) phospho-Tau with AT100 in frontal cortex (C) and hippocampus (D) of aged biGT mouse [32]. (E) Amyloid with 3D6 in cortex of aged APP.V717I mouse [42,49]. (F) Confocal microscopy of amyloid (red) and neurofibrillary tangles (green) in brain of biAT mouse as model for combined AD pathology [32]. Scalebars all 100 μ m, except F 50 μ m.

concentration but even more so by relative levels of different amyloid peptides [44–46]. An important contribution is assigned to ApoE-lipoproteins in the regional distribution of amyloid in mice [47] and in AD-patients [48]. This must be analyzed in relevant mouse models to understand the contribution of ApoE to the overall AD problem, an aspect that is outside the scope of this review.

3.3. Amyloid mice lack tauopathy and neurodegeneration

The most marked feature of all APP transgenic models with amyloid pathology is their complete lack of authentic tauopathy. At best, phosphorylation of endogenous mouse protein tau in dystrophic neurites surrounding amyloid plaques was observed in brain of APP mice, but no fibrillar tau aggregates or tangles [27,29,49]. Tauopathy was not considered obligatory in an AD model, nor expected per se, although predicted by the amyloid cascade hypothesis that should apply also to mouse models. The cognitive normal individuals with high amyloid load identified by PIB-PET imaging, are then predicted to still lack tauopathy and thereby escape dementia. These individuals do not qualify as AD-cases but are interesting to define the genetic make-up that prevents amyloid pathology to induce tauopathy. Whether this is a temporary condition, with tauopathy merely delayed, or a permanent state makes these “amyloid-escapees” not less interesting.

The lack of tauopathy in APP transgenic models is a drawback, but an even greater setback for the amyloid cascade hypothesis. The “amyloid-only” condition does not elicit tauopathy and thereby fails to produce AD, which is defined as the combination of both pathologies. This problem was and is insurmountable for all model-makers who failed to deliver a complete pathology model for AD by expression of mutant or wild-type APP in mouse forebrain neurons *in vivo*. The conundrum was eventually solved in bigenic and triple transgenic mice, by the extra incorporation of mutant tau transgenes, which evidently does not conform to the genetic situation of any AD patient.

On the other hand, the available “amyloid-only” mice have been and remain highly informative for the differential analysis of various molecular, systemic and pathological aspects of the generation of the amyloid peptides, of their physiological and pathological contributions and repercussions on cognition and on AD-related defects in adult and ageing mouse brain. We have used our APP.V7171 mice extensively in that respect, including many combinations with transgenic and knock-out mice to probe the contribution of relevant genes, *i.e.* presenilin1/2, BACE, ADAM10, ApoE4, Tau, A2M, GSK3, among others [27–33].

Straightforward, obligatory characteristics of AD models are defects in behavior and cognitive performance. Various cognitive tests used to evaluate transgenic APP mice range from the water-maze, to other types of mazes, over fear-conditioning and novel object recognition tasks. Overall, APP mice exhibit more or less strongly impaired cognition, although not consistent or similar in all paradigms in all models. The general conclusion is that an overload of amyloid peptides and/or APP-metabolites negatively affect synaptic functioning and plasticity. One important variable, however, is the age of onset of the cognitive deficits, *i.e.* before, concomitant with or after the deposition of amyloid plaques. In APP.V7171 mice, we have dissociated the early cognitive defects from the later amyloid plaque pathology, which was the first report that stepped away from amyloid plaque deposition as the cause of impaired cognition in a living AD model [49]. The venue proved attractive for the development of alternative views on the cognitive decline in AD and for a revised or extended amyloid cascade hypothesis [5].

The issues surrounding amyloid peptides and plaques remain food for academic debate and more. Elimination of excess amyloid peptides from neurons and synapses by deposition as parenchymal plaques could be a first means of protection. This can however not be a long-term effective strategy because increasing physical protein deposits in any organ can never be an efficient route of elimination, as demonstrated by

atherosclerotic plaques, gall and kidney stones, immunoglobulin light chain aggregates, serum amyloid, and many other instances.

Importantly, the observation of the early phenotype in APP.V7171 and other amyloid mice effectively dissociated amyloid plaques as the direct cause of cognitive demise. The implication is that impaired cognition must be attributed to amyloid peptides proper, in chemical or physical forms other than plaques. The hypothesis has now gained wide acceptance and experimental support in the identification of A β -oligomers as responsible for functionally disturbing synaptic transmission, also *in vivo*. Nevertheless, the actual composition and number of peptides, their molecular nature and conformation in the presumed faulty A β -oligomers, are all but well known. Nor do we understand yet their mode of action *in vivo*, nor the receptors to which they bind specifically or not, notwithstanding the considerable progress in describing their effects on cognitive parameters *in vivo*. This brief paragraph constitutes a dense concentrate of the problems that overshadow the amyloid-cognition-AD relation. The issues are vast and surpass the scope of this review.

Finally, despite tentative claims to the opposite, “amyloid-only” transgenic mouse models do not suffer substantial neurodegeneration in limbic regions, not even at old age, which sets them apart from AD patients. Ageing is evidently a relative issue, particularly in mice. Neurodegeneration typified by death of neurons is responsible for the progressive brain atrophy in later stages of AD, which could be merely a matter of time inherent to the longer duration of the disease in humans. Consequently, only initial stages of AD can be recapitulated in mice and perhaps we should aim only for that in mouse models. Alternatively, neurodegeneration can be recapitulated by other means and in other types of models, which we have approached previously in a conditional p25 mouse model [50] and most recently in AAV-Tau based models [34]. The AAV-based models unexpectedly provided important clues to the responsible action of wild-type Tau4R and to the mechanisms involved, as discussed below.

3.4. Tau transgenic mice, models for Tauopathy and neurofibrillary tangles

Available transgenic mice that model tau-mediated neurodegeneration rely on the expression of wild-type tau isoforms or of mutants implicated in familial tauopathies. The variable success and the problems are reviewed in detail [24,25,27,28,37–39,51,52]. Here, we limit our overview to selected models with explicit tauopathy that are informative for the questions raised in the previous sections.

Transgenic mice that express a single isoform of wild-type human Tau ranged from the shortest Tau3R/ON to the longest Tau4R/2N. Most markedly, mice that express wild-type tau develop motor problems that are associated with axonopathy. The eventual brain and spinal cord pathology usually includes aberrant phosphorylation but never robust and authentic tauopathy defined as aggregates, fibrils and tangles. Noteworthy, introduction of the entire human MAPT gene in the mouse genome led to expression of all six human tau isoforms in neurons [53,54]. Eventually, redistribution of protein Tau from axons to cell bodies led to formation of tau aggregates and cell death at very old age. Extremely interesting was the observation that degenerating neurons did not contain tau aggregates, explicitly interpreted by the authors to “... suggest that cell death can occur independently of NFT formation” [54].

Tau4R mice we have generated followed the rule: severe axonopathy developed first in hindbrain, brainstem and spinal cord, resulting in severe claspings of hind legs. In homozygous Tau4R offspring this was already obvious at weaning [55]. The axonopathy was typified pathologically by axonal dilatations, leading to wallerian degeneration and muscle wasting [55]. The phenotype was aggravated by treatment of Tau4R mice with lithium salts, and rescued completely by co-expression of GSK3 β in bigenic mice [56]. These data not only identified this important role of GSK3 β , but was also the first experimental demonstration of GSK3 β as efficient tau-kinase in brain *in vivo* [56].

Nevertheless, despite the evident increased pool of protein Tau4R phosphorylated at crucial phospho-epitopes by GSK3, the bigenic Tau4RxGSK3 β mice did not develop tauopathy. We concluded that GSK3 is essential but not sufficient tau-kinase in neurons to cause tauopathy *in vivo* [56], subsequently borne out in the biGT mice [32,33].

Transgenic mice expressing protein mutant tau associated with FTDP-17 are in general successful in producing authentic tauopathy with neurofibrillary tangles, eventually even neurodegeneration [24,25,28,37–39,51,52]. We generated Tau-P301L transgenic mice that develop profound tauopathy, that was moribund in contrast to the Tau4R mice, despite very similar spatial and temporal expression at similar protein tau levels [55,57]. Their typical age-dependent cognitive and motoric defects have been observed also in other mutant tau transgenic mouse strains, resulting in authentic tauopathy although variable in intensity and in brain-region in expressing mutant tau, e.g. Tau-P301L [57–61], Tau-R406W [62–64], Tau-V337M [65,66], Tau-P301S [67,68], Tau-N279K [69] (reviews [37–39]). Nevertheless, tauopathy in these mouse models must be considered closer to FTD than to AD tauopathy, which imposes restrictions when used in pre-clinical tests of treatments specific for AD.

4. Newer models, newer views

4.1. Bigenic and multiple transgenic mice as more complete AD-models

Because single transgenic mice failed to recapitulate comprehensively the complete AD pathology, the development of multiple transgenic mice was regarded essential to gain deeper insight in the pathogenesis of AD. Most early models of this type were combinations of mutant APP and mutant PS1 that have been instrumental to delineate the contributions of γ -secretase activity to amyloid peptide generation and amyloid pathology. This overview is not primarily concerned with those aspects and models, but remains concentrated on the relation amyloid-tau pathology.

The most multigenic mouse model for AD co-incorporates minigenes coding for mutant APP.SW and for Tau.P301L in the genome of PS1.M146V knock-in mice. These Triple tg or 3xTg mice progressively present with amyloid pathology, first intracellularly followed by diffuse and eventually senile plaques, alongside tau phosphorylation ending in tauopathy with neurofibrillary tangles [39,70].

Similar to our APP.V717I mice, the 3xTg mice suffer cognitive impairment and problems with memory, synaptic dysfunction and diminished LTP before the deposition of amyloid plaques [39,70]. These defects correlate with the accumulation of intraneuronal A β , again like in APP.V717I mice [42,49]. Immunotherapy of Triple tg mice with an amyloid-based vaccine diminished the amyloid pathology but also the early, although not the late tau pathology, which was claimed to problems with proteasomal degradation [39]. The amyloid cascade hypothesis appeared to be respected because the data indicated that tangle pathology was subordinate to or a consequence of amyloid peptides.

Somewhat unfortunately, the strategy used to generate the 3xTg mice prevents direct comparison to single transgenic parental mouse strains. That type of comparison was used proficiently in the bigenic model combining JNPL3(TauP301L) x Tg2576 (APP.SW) mice. These bigenic mice develop amyloid deposits like the parental Tg2576 mice and exhibit tauopathy even in excess of the parental JNPL3 mice [58,71]. We crossed our Tau.P301L mice that develop moribund tauopathy [57,60] with our APP.V717I mice that develop amyloid pathology [44,49]. The resulting biAT mice present robust amyloid and tau pathology with amyloid plaques in forebrain and NFT in pyramidal neurons of hippocampus and cortex, starting at age 10–12 months and becoming established as AD-like pathology around age 13–15 months, comprising diffuse and senile amyloid plaques, intra-neuronal tangles in cortex and hippocampus, amyloid angiopathy and inflammation [32].

The detailed time-line of brain pathology and biochemistry of the biAT mice and the associated phenotypical and cognitive features relative to the parental single transgenic mice is ongoing. The amyloid pathology in biAT mice is very similar in aspects and timing, but more intense, as in the parental APP.V717I mice that lack overt tauopathy [27,42,44,49]. In contrast, the tauopathy of biAT mice is dramatically enhanced in the hippocampus and cortex relative to the parental Tau.P301L mice. These data expose the marked synergism between the two major AD-pathologies in the limbic system of the biAT mice [32,33]. Surprisingly, the moribund brainstem pathology typical for the Tau.P301L mice, is largely alleviated despite the combined AD-like pathology, with a marked increase in the life-span of the biAT mice. Somewhat serendipitously, the underlying mechanism was highlighted by the bigenic Tau.P301L x GSK3 β -S9A mice, based on the same Tau.P301L strain in combinations with the overexpressing GSK3 β mice, characterized before [56,57]. The biGT mice develop the most dramatic forebrain tauopathy reported in any transgenic model [32]. NFT accumulate massively in nearly all cortical and hippocampal neurons that become packed with filamentous and fibrillar aggregates in ageing biGT mice of 15–18 months old (Fig. 1). They provide pathological and biochemical evidence that GSK3 β contributes essentially to phosphorylation of protein tau *in vivo*, causing tau-aggregation in neuronal cell-bodies and processes, causing neuronal dysfunction, although not neuronal death [32,33].

Beginning amyloid pathology already activates both GSK3 isozymes by tyrosine phosphorylation in young APP.V717I mice [32,33]. Thereby, both GSK3 isozymes contribute to increased phosphorylation of protein tau, and eventually feed back onto APP processing. Remarkably, however, the resulting pathological outcome differs in different brain-regions. Both biAT and biGT mice have increased tauopathy in limbic regions, but both show alleviation in brainstem and spinal cord relative to the parental Tau.P301L mice [32,33,72]. The positive effect of amyloid and GSK3 on survival in biAT and biGT mice is attributed to the markedly diminished tauopathy in the autonomic control centers in the brainstem. This hypothesis is being substantiated *in vivo*, by electrophysiological analysis of the brain-stem and by plethysmography [72].

Time-line analysis demonstrates that the early activation of both isozymes in the parental APP.V717I mice correlates with increased phosphorylation of tau already at young age [32]. Consequently, the correlated appearance of intracellular A β , activated GSK3 isozymes (tyrosine phosphorylation) and increased tau-phosphorylation impinge on the cognitive defects also evident at young age [49].

The data underline that amyloid is up-stream of tauopathy *in vivo*, in support of the amyloid cascade hypothesis. Note, however, that the APP.V717I and biGT mice are in fact EOFAD models, which leaves the quest for factors upstream of amyloid pathology in late onset and sporadic AD open. The models further underline the important contribution of GSK3 β , and possibly of, the GSK3 α isozyme, following their increased tyrosine phosphorylation in young APP.V717I mice. GSK3 isozymes can become phosphorylated on Y279/Y216 by auto-phosphorylation or by tyrosine kinases such as Fyn or Abl. The actual mechanism, and the effect of amyloid, remains unexplained and unexplored *in vivo*.

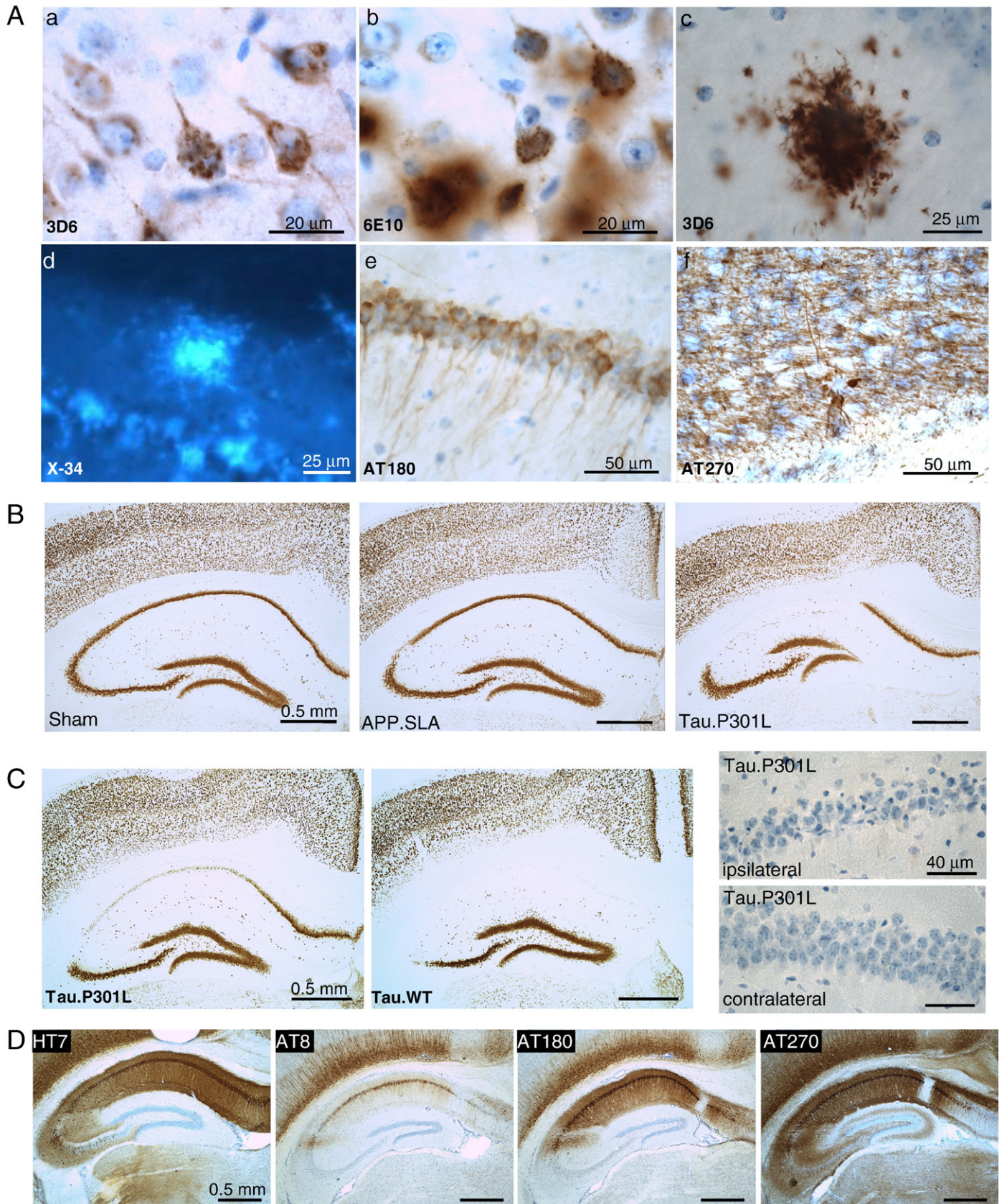
In conclusion, the comparative analysis of the two bigenic strains, biAT and biGT, revealed the striking similarity in the progression of tau phosphorylation and tauopathy, and in the resulting clinical outcome. The data position GSK3 as central player in the complex web that relates amyloid and tau pathology. Whether this also reflects a physiological link remains to be unraveled experimentally.

4.2. Viral AAV-APP.SLA model: amyloid pathology without neurodegeneration

Over the last decades invaluable insight in the molecular, physiological and pathological aspects of AD-related proteins has been

gathered from numerous studies on different types of single and multiple transgenic mice, outlined in previous sections. The efforts needed to generate, select and characterize the most informative

models that we now retain, have been substantial while their implementation in pre-clinical studies requires major efforts. These and other reasons, including pressure from patients and relatives, and



from society at large to find disease-modifying remedies, incited us and others to explore alternative options for more rapid and flexible models. Viral vectors are attractive to express proteins of interest in defined brain regions, both experimentally and for gene therapy in humans. Here we discuss in brief recent developments and findings in a model that recapitulates the important feature of tauopathy, i.e. tau-mediated neurodegeneration, which is largely lacking in transgenic models.

Adeno-associated virus (AAV) vectors have been proposed for gene delivery in CNS because of the safety and unique aspects. Most humans are populated with AAV and the virus persists in humans for long periods without apparent negative consequences. Recent technical progress has improved some earlier shortcomings of AAV as experimental vectors and graded them up to an excellent delivery vehicle in experimental medical research [73,74]. We first aimed to use AAV vectors to recapitulate AD-like pathology in wild-type mice. AAV-particles with chimeric capsids of serotype 1/2 express the embedded transgenes under the control of the human synapsin1 gene promoter in pyramidal hippocampal neurons *in vivo* [75,76].

Wild-type mice were injected intracerebrally with AAV-particles and robustly expressed either wild-type human APP or with a designed APP.SLA triple mutant, harboring the Swedish, London and Austrian mutations. The APP.SLA mutant, but not wild-type APP caused progressive accumulation of APP-metabolites including amyloid peptides in pyramidal neurons in hippocampus CA1/2-regions and in deep layers of the cortex. From 3 weeks post-injection (p.i.) onwards, intracellular amyloid accumulated in vacuolar and granular bodies in CA1/2 pyramidal neurons, evolving to diffuse, cell-associated amyloid accumulations. Eventually at 6 months p.i., authentic amyloid plaques were deposited over the hippocampal area and nearby cortex (Fig. 2). Interestingly, amyloid pathology was associated with increased phosphorylation of endogenous protein mouse tau at residues T181 and T231, which phospho-epitopes defined by antibodies AT270 and AT180, respectively. Loss of neurons remained marginal, even at 6 months p.i. of AAV-APP.SLA when increased phosphorylation of protein tau was evident [34]. When not wild-type but young Tau.P301L mice were injected with AAV-APP.SLA, not only amyloid plaques but also tau-aggregates developed at 6 months p.i. (unpublished observations). These and ongoing studies are needed to complement and extend observations with lentivirus-mediated expression of Tau.P301S in APP23 mutant mice [77]. The novel AAV-based tau and amyloid models corroborate observations in transgenic amyloid mouse models that recapitulate the amyloid pathology of AD with only minimal or no neurodegeneration [49].

4.3. Viral AAV-Tau model: pyramidal neurodegeneration without tauopathy

In sharp contrast, very similar manipulations to express either wild-type Tau4R or mutant Tau.P301L resulted in extensive neurodegeneration and loss of pyramidal neurons in CA and adjacent cortical layers already at 3 weeks p.i. (Fig. 2). Interestingly, a very similar outcome was observed with AAV-Tau4R, extending the neurotoxic effect to wild-type human Tau4R. Both observations were unexpected, and analyzed in widely differing directions to gain insight in the mechanism of neuronal death in this model.

Firstly, neurodegeneration was not due to massive overexpression of human protein tau. The actual levels in hippocampal extracts were near-physiological and only about twice those of endogenous mouse Tau4R. This was similar to the AAV-APP.SLA model, where hippocampal human APP.SLA were about twice those of endogenous murine APP, but which failed to cause neurodegeneration. Expression of EGFP by a similar AAV-vector proved harmless to pyramidal neurons over long time-periods [34]. The data excluded that overexpression of any protein by these vectors caused the observed neurodegeneration, and qualifies the observed pyramidal cell-death as specific for human protein tau. Because the conclusion holds also for wild-type Tau4R, the model conforms to observations in familial FTD: intronic and exonic mutations cause similar clinical outcome by expression of wild-type or mutant Tau4R, respectively (cf background Section 1.4). The contribution of the tau-mutations to the mechanism of tau-neurotoxicity awaits a rational explanation in human FTD patients.

Secondly, intracerebral injection of 3- or 10-fold lower doses of AAV-Tau with consequently lesser expression of human protein tau, produced more graded and less rapid loss of hippocampal neurons. The AAV-tau induced neurodegeneration is thereby further demonstrated to be directly caused by, and proportional to the near-physiological levels of protein tau.

Thirdly, protein Tau became phosphorylated at many pathological epitopes, with AT8, AT180, AT270 as most evident. But increased phosphorylation did not lead to appreciable deposition of Tau-aggregates in the degenerating pyramidal neurons before they were lost. Biochemically, more loose aggregates were observed by western blotting following non-reducing SDS-PAGE. High Mr tau-containing complexes migrated in an extensive smeared pattern, that resolved into the normal banding pattern of protein tau (~64–70 kDa) upon disulfide bond reduction [34]. These high Mr complexes apparently are disulfide bonded and partially SDS-resistant, which is further examined biochemically.

Interestingly, preliminary experiments with a similar expression of a C-truncated version of wild-type protein tau, lacking the entire microtubule binding domain, proved harmless and did not induce neurodegeneration. The inherent implication that microtubule binding of protein tau is essentially involved in the degenerative mechanism, oriented our further analysis to mechanisms that involve microtubuli, i.e. axonal transport and cell cycle. The former is of paramount importance for neurons, while the latter is an evident problem for post-mitotic neurons.

Also of note, microgliosis was intense and spatially and temporally closely associated with AAV-Tau mediated degenerating neurons [34]. This was most recently also reported in a similar rat model aiming at a different pathology in a different brain-region [78]. We observed previously a very similar correlation in an unrelated transgenic model for hippocampal sclerosis, with neuronal death provoked by inducible expression of p25, the truncated activator of cdk5 [50]. Also in that model the intense neurodegeneration and brain atrophy was not accompanied by aggregation of protein tau. The data confirmed that cdk5 activated by p25 or p35 is not an effective tau-kinase in mouse brain *in vivo* [50,79]. In yeast models we actually observed the opposite, because not overexpression but inactivation of the pho85 kinase, the yeast orthologue of mammalian cdk5, strongly promoted phosphorylation and aggregation of tau [80].

Fig. 2. Intracerebral injection of AAV-vectors in wild-type mice: neuro-degeneration without tangles by AAV-Tau and amyloid pathology without neurodegeneration by AAV-APP.SLA. Intracerebral injection in wild-type mice of AAV vectors analyzed 3 to 24 weeks post-injection. (A) Various stages of amyloid pathology in AAV-APP.SLA injected mice, panel a: intracellular amyloid at 3 weeks p.i. (antibody 3D6). Scale bar 20 μ m. panel b: additional extracellular diffuse amyloid at 12 weeks p.i. (antibody 6E10). Scale bar 20 μ m. panel c: amyloid plaques at 24 weeks p.i. (antibody 3D6). Scale bar 25 μ m. panel d: amyloid plaques at 24 weeks p.i. (histology with compound X-34). Scale bar 25 μ m. panels e, f: phosphorylated mouse tau at 24 weeks p.i. Scale bars 50 μ m. (B) Pyramidal cells in hippocampus at 24 weeks p.i. (antibody NeuN) in wild-type mice injected with AAV-APP.SLA or AAV-Tau.P301L mice or sham-injected. Scale bars 0.5 mm. Note normal appearance in AAV-APP.SLA injected mice, except for small section in CA2 as opposed to severe neuro-degeneration in CA and cortex of AAV-Tau.P301L injected mice. (C) Similar neurodegeneration in AAV-Tau.P301L and AAV-Tau.4R injected mice at 3 weeks p.i. (NeuN antibody). Scale bar 0.5 mm. Right panels: hematoxylin-staining reveals neurons with degenerating nuclei in AAV-Tau injected mice at 3 weeks p.i.. Scale bars 40 μ m. (D) Expression of human Tau (antibody HT7) and selected phospho-epitopes (antibodies AT8, AT180, AT270) in AAV-Tau.P301L injected mice at 3 weeks p.i.. Scale bars 0.5 mm.

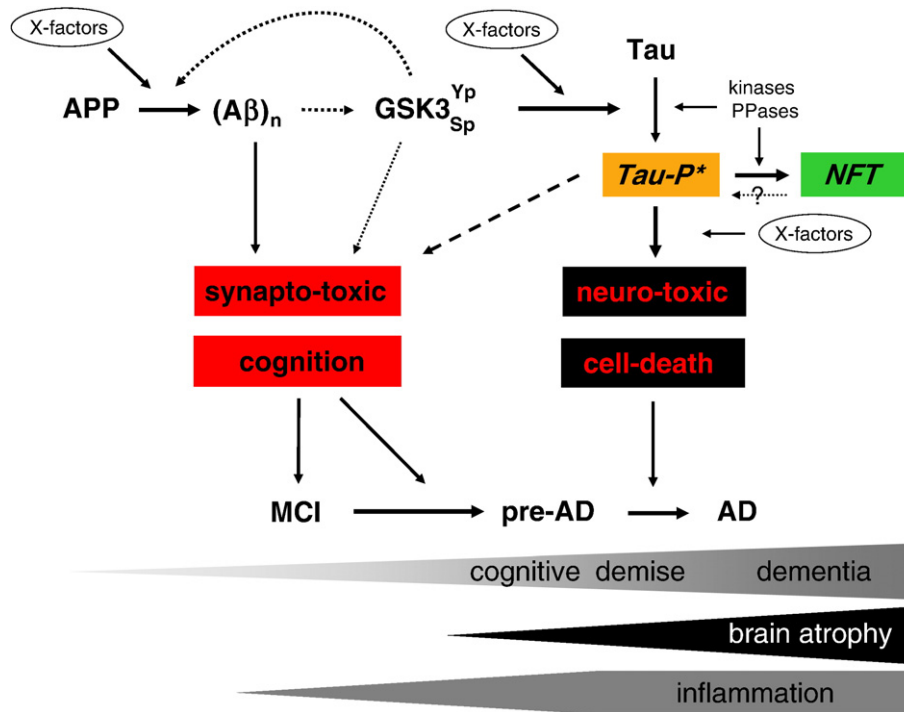


Fig. 3. Schematic representation of inter-relations between APP/amyloid, GSK3 and protein tau. The central incorporation of GSK3 α/β , with inhibitory S21/S9 and activating Y279/216 phosphorylations, is discussed in the text, based essentially on our findings in single and bigenic mice [32]. The various connections are primarily distilled from *in vivo* observations in transgenic mice and represent proven (solid arrows) or proposed effects (broken arrows). Tau-P* represents the hypothetical intermediate form of phosphorylated protein tau in a transitional conformational state that directs it either into aggregation or towards neuronal toxicity. The orange colored Tau-P* box reflects its proposed ambivalent status in provoking neurotoxicity or aggregation, i.e. danger or relative safety. The resulting formation of neurofibrillary tangles (NFT) is purposely meant to be a neuronal safety measure, which is reflected by its green background. NFT-formation constitutes a temporary solution to reduce levels of Tau-P*, but can evidently not be considered a long-term solution, as proven in tauopathy patients. The molecular factors and mechanisms behind the decision of neurons to develop tauopathy as NFT rather than suffer neurodegeneration and cell death, is not understood as discussed in the text. The contribution of kinases and phosphatases is evident, as opposed to that of extraneous X-factors that are various and many, i.e. genetic, epigenetic, environmental, nutritional, educational, social, ... The unknown factors can act on many of the shown relations and connections, but for clarity we included them only at the most intricate control points in the scheme. The Tau-P* intermediates are the central molecular species in the overall process, acted upon by the additional unknown factors that can vary both in origin and in type, i.e. neuronal, microglial, mitochondrial, cell-cycle control kinases, protein-folding chaperones, heat-shock proteins, cytokines, growth factors,.... The impact of various different actors leads to various and variable clinical and pathological symptoms, as observed in AD and particularly in tauopathy patients. A tentative time-line from mild cognitive impairment (MCI) to the ultimate deep Alzheimer dementia typified by severe brain atrophy, is suggested schematically, not taking into account the deposition of amyloid plaques, because we consider these as cognitively inert, at least initially, as discussed.

5. Concluding remarks and prospects

We have schematically summarized the combined findings in different mouse models to depict the various players and their interrelations (Fig. 3). The main axis conforms with the amyloid cascade hypothesis, whereby accumulation of amyloid is the main trigger in AD and leads to tauopathy. Central in this scheme, and in our work-hypothesis, is GSK3 supported by the finding that amyloid activates both GSK3 isozymes by tyrosine phosphorylation, and these contribute essentially to tau-phosphorylation [32,33]. The important inter-connections in the scheme are based on *in vivo* observations in transgenic mice, as discussed in foregoing sections. Note that GSK3 is linked to cognition by its other, inhibitory type of phosphorylation at Serine9 [81].

The data allow to propose that the differential phosphorylation of protein tau is directly linked to the differential pathological outcome, either slow aggregation or rapid degeneration. On the one hand, severe neurodegeneration is evident without appreciable tau-aggregation in the AAV-Tau and p25 models [34,50]. On the other hand, tau-aggregation and tauopathy is not accompanied per se by marked neurodegeneration in transgenic biAT and biGT mice [32,33]. We pose that any imbalance in the physiological activities exerted by Tau and GSK3 causes functional deficits that eventually lead to synaptic, axonal and neuronal degeneration, as observed in models *in vivo* (reviews [82]) and sustained by genetic evidence [83].

Based on the combination of models, we further defend the thesis that neurodegeneration is the outcome of dual actions, beginning

with endogenous damage by mal-formed proteins like protein tau, or α -synuclein, huntingtin, undigested or undigestible proteins in storage diseases, cholesterol-transport proteins, and others. A second factor is nevertheless as essential, which can be neuronal or microglial, related to oxygen-supply, glucose-overload or shortage, cholesterol metabolism or other essential metabolites. This thesis finds support in observations by us and others, referred to throughout this review. Moreover, the data now convincingly demonstrate that large protein-aggregates are not the direct nor even essential cause of neuronal death, illustrated again by recent observations that tangle-bearing neurons survive despite membrane damage due to tauopathy [84]. These propositions imply a larger number of potential combinations of damaging factors that in turn dictate the number of ways a neuron can die, a problem not yet solved [85]. The data thereby still do not answer the “chicken-and-egg-question” in tauopathy: phosphorylation, aggregation, neuro-degeneration [24].

In the scheme (Fig. 3) the Tau-P* acronym represents a hypothetical, unidentified intermediate form of phosphorylated protein tau, either a single molecule or a small aggregate that is either not bound to microtubuli, or bound in an abnormal aggregated mode [80]. Tau-P* most could mark a transitional, but also a more stable conformational molecular state or form. These elusive Tau-P* molecules that we propose are faced with two options: aggregate or not, with similar final outcomes but different time-lines. The first option is to aggregate rapidly and thereby to be effectively neutralized as neurotoxic factor. Nevertheless, tau-aggregates solve the problem only temporarily because over a longer period of time, tau-aggregates

cause neurons to die, giving rise to ghost tangles. This is the classic concept in the tau-field.

Alternatively, when aggregation is prevented by X-factors (Fig. 3) or not prompted, the Tau-P* species persist and cause cellular damage from within neurons, or from the outside after secretion, by as yet unknown mechanisms. We realize that these actions and mechanisms are counter-intuitive and not conform accepted cell-biological dogmas. They comprise and involve several unresolved issues, not in the least the molecular logic that underlies the decision made by neurons to develop either tau-aggregates or succumb. The Tau-P* intermediates are proposed to play a central role in this process, evidently acted upon by factors that can vary in origin from activated microglia to damaged mitochondria, over cell-cycle control kinases and heat-shock proteins. The relative spatial and temporal impact of these different players then produces the various clinical symptoms and pathological hallmarks, typical for tauopathies and AD. These competing or complementing processes define eventually the timeline and progression from mild cognitive impairment to deep dementia with severe brain atrophy, typical for the terminal stage of Alzheimer's disease (Fig. 3).

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