● PERSPECTIVE

Neurofibrillary tangles in Alzheimer's disease: elucidation of the molecular mechanism by immunohistochemistry and tau protein phospho-proteomics

As a key contributor to memory storage, the synapse is one of the earliest affected neuronal components in Alzheimer´s disease (AD). Under physiological conditions, the synaptic connections between neurons undergo activity-dependent functional and morphological re-organisation. This dynamic, 'plastic' neural ability critically depends on the structural integrity of the synapse. Thus, proteins that are implicated in preserving the organisation and dynamics of synaptic connections, including microtubules of the cytoskeleton and associated proteins, have attracted much focus for their involvement in the malfunctioning AD synapse.

Neurofibrillary tangles (NFTs) are a fundamental neuropathological hallmark of AD. NFT-bearing neurons have been characterised by loss of cytoskeletal microtubules and tubulin-associated proteins. Although the exact molecular mechanisms linking the loss of cytoskeletal elements to NFT development remain unclear, signal transduction pathways involving protein phosphorylation and de-phosphorylation are likely to play a central role in the formation of neurofibrillary lesions (**Figure 1**). For instance, the major constituent of NFTs, the microtubule-associated protein tau (Mapt), is abnormally phosphorylated (hyperphosphorylated) in the brains of AD patients compared to non-demented individuals (Zempel and Mandelkow, 2014). Hyperphosphorylation occurs in an epitope-specific manner during the course of AD, and is known to underlie the missorting of tau from a primarily axonal to a somatodendritic location in neurons. This alteration is thought to contribute not only to the deregulation of microtubule dynamics, but also to tau polymerisation and aggregation (Alonso et al., 2008).

Conceivably, investigating the molecular mechanisms behind the causes and consequences of Mapt hyperphosphorylation may lead to an improved understanding of the protein's role in NFT formation, and further elucidate the connection between NFT deposition, synaptic loss and memory impairment in AD. Interestingly, formation of NFTs and synapse loss are correlated during the course of AD (Gomez-Isla et al., 1997) and each of these processes further correlates with cognitive decline, arguing that these AD lesions occur in a related, rather than independent manner.

Immunohistochemistry of phospho-sites related to Mapt in human *post-mortem* brain and in mice with amyloid pathology: The regional progression of neurofibrillary lesions is highly stereotyped in the AD brain. In their landmark study of 1991, Braak and Braak used *post-mortem* tissue from demented and non-demented individuals to summarise three stages of propagation of tau-related pathology (Braak and Braak, 1991). In the Braak staging system, NFTs appear initially in the trans-entorhinal region of the temporal lobe, spreading to limbic areas such as the hippocampus, and finally affecting large areas of the neocortex.

In addition to the region-specific spread of NFTs, distinct morphological stages of NFT formation have been also discerned in the AD brain. Each individual stage has been associated with characteristic patterns of Mapt hyperphosphorylation, suggesting that specific phosphorylation events may contribute to the evolution of tau pathology (Augustinack et al., 2002). Thus, pre-NFTs are typically observed as punctuate or diffuse tau inclusions within the cytoplasm of an otherwise normal-looking neuron, and are preferentially labelled by antibodies to tau phospho-epitopes pSer262, pThr153 and pThr231. Mature NFTs, most intensely stained with antibodies to tau epitopes of pThr175/181, pSer46, pSer214, pSer262/pSer356 and pSer422 are compact filamentous aggregates of cytoplasmic tau that invariably displace the cell nucleus towards the periphery of the soma. Extracellular, 'ghost' NFTs result from the death of the host neuron, comprising substantial amounts of filamentous tau protein, and are recognized by the absence of a stainable nucleus. Extracellular tangles are preferentially stained with antibodies AT8 and PHF1 recognizing pSer202/pThr205 and pSer396/pSer404, respectively. The fact that the phosphorylation of specific tau epitopes corresponds to distinct stages of NFT formation clearly points towards a sequence of early *vs*. late phosphorylation events, underlying the initiation and progression of tau lesions in AD. Indeed, it has been proposed that phosphorylation at Thr231 is an initiating event for the formation of NFTs, followed by oligomeric tau aggregation, filament formation, and neuronal cell death (Lasagna-Reeves et al., 2012).

The characterisation of tau pathology has been performed in several mouse models of amyloidosis, albeit not in an exhaustive manner. Although the general consensus is that amyloidosis models do not recapitulate AD tau lesions in terms of NFT formation, amount and propagation, a growing body of preclinical studies has shown amyloid beta-induced phosphorylation of endogenous mouse tau *in vivo*, using antibodies to the aforementioned tau epitopes and techniques such as immunohistochemistry and western blotting (Stancu et al., 2014).

Several reasons might explain why the site-specific phosphorylation of tau in amyloidosis models does not translate to robust neurofibrillary pathology, as in AD patients. First, adult mice are known to express fewer tau isoforms than humans (three *vs*. six), and are perhaps less liable to increases in endogenous tau phosphorylation. Second, the mouse lifespan might be too short for the complete sequence of neurofibrillary pathology to unfold in models of amyloidosis. Third, unidentified phosphorylation sites and/or additional post-translational modifications (PTMs) of endogenous mouse tau may render this species resistant to the development of NFTs.

Phospho-proteomics to characterise tau-related lesions in amyloidosis models: Only a handful of studies have assessed tau PTMs in mouse models of amyloidosis using mass spectrometry. In a comprehensive study of amyloid precursor protein (APP) transgenic mice harboring the human Swedish and Indiana mutations (J20 line), Morris et al. confirmed 41 and identified 22 novel tau modifications, including phosphorylations, methylations, acetylations and ubiquitinations (Morris et al., 2015). The majority of altered phosphorylation sites of Mapt were adjacent to the microtubule-binding repeat domain, and were comparable in number with phosphosites identified from wild-type mice or *post-mortem* brain tissue from non-demented humans (Funk et al., 2014). These results indicate that the number of phosphosites of endogenous tau is similar in wild-type mice, J20 transgenic animals and cognitively healthy individuals. Interestingly, AD 'diagnostic' tau epitopes, including pThr181, pThr231, pSer202 and pSer404 were commonly detected in both control mice and non-AD humans, suggesting either that additional tau modifications are involved in the formation of NFTs

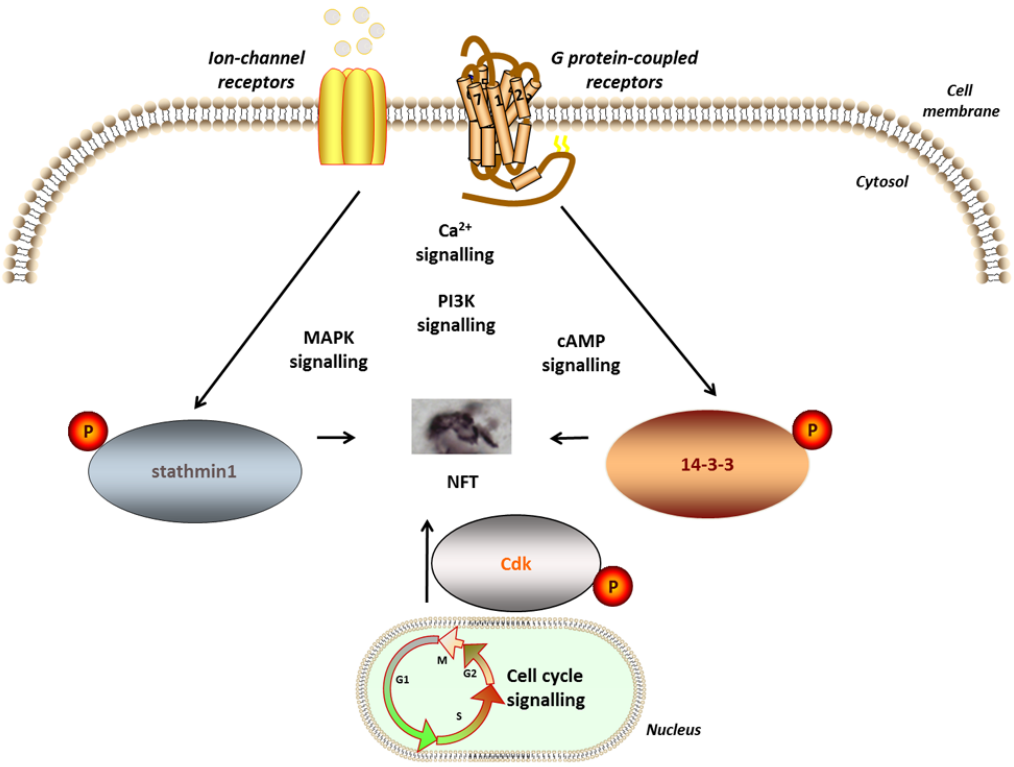

Figure1 Neuronal phospho-signallingand tangle formation.
The figure shows a post-synaptic neuron with its nucleus. After stimulation of ion-channels and/or G protein-coupled receptors *via* neurotransmitters originating from the presynapse, phospho-signalling associated with long-term potentiation (activation of phosphorylation) or long-term depression (suppression of phosphorylation), including Ca^{2+}-, MAPK, PI3K or cAMP-mediated signalling is activated/inhibited. This can lead to changes in the phosphorylation status of down-stream targets such as stathmin1 or 14-3-3 proteins, which may in turn induce an altered phosphorylation profile of tau protein that can eventually culminate in NFT formation. Neuronal cellular metabolism is also important in this process, where cell cycle signalling may lead to an increased phosphorylation of Cdk proteins affecting NFT formation. Phosphorylation defects in stathmin1, 14-3-3 and Cdk (such as Cdk5) proteins have been shown to be involved in Alzheimer´s neuropathology. cAMP: Cyclic adenosine monophosphate; Cdk: cyclin-dependent kinase; MAPK: mitogen-activated protein kinases; NFT: neurofibrillary tangles; PI3K: phosphoinositide 3-kinase. The figure was generated by images from commercial software (http://www.visiscience.com).

or/and that the excess phosphorylation of the aforementioned epitopes, rather than phosphorylation *per se*, underlies the development of NFTs.

Unlike J20 mice, our integrated quantitative phospho-proteomics study in 12 months old APP_{swe}/Presenilin 1 $(PS1)_{\Delta E9}$ male transgenic mice demonstrated differences in tau phosphorylation compared to age-matched controls within four selected brain regions (Kempf et al., 2016). The levels of multiple phosphopeptides, corresponding to sites Thr58, Ser688/Thr695, Ser506, Ser494, Ser696, Thr473 and Thr523 of the canonical tau sequence in the olfactory bulb, Ser688/Thr695 in the neocortex, Ser494 and Thr58 in the brainstem, and Ser688/Ser696, Ser490/Ser491 as well as Ser688/Ser692/Ser696 in the hippocampus, were altered in $APP_{swe}/PS1_{\Delta E9}$ mice compared to wild-type littermates. Of the aforementioned peptides, only Ser506 is a known phosphosite of Mapt reported by the PhosphoSitePlus database. While increased levels of phosphorylation were observed for Ser688/Thr695 in the $APP_{swe}/PS1_{\Delta E9}$ cortex, hippocampal phosphopeptides were decreased compared to control, and a mixed picture of both increases and decreases in peptide phosphorylation were observed in the transgenic mouse olfactory bulb and the brainstem. For example, levels of Mapt-Thr58 were higher in the olfactory bulb, but lower in the brainstem of $APP_{swe}/PS1_{\Delta E9}$ mice compared with wild-type animals. In contrast, levels of Mapt-Ser494 were increased in the brainstem, but reduced in the olfactory bulb of transgenic *vs*. wild-type mice. It is intriguing to speculate that the phosphorylation changes on MaptThr58 and Ser494, which occurred in opposite directions in the olfactory bulb and brainstem of $APP_{swe}/PS1_{\Delta E9}$ mice, may be associated with the presence and absence of Gallyas-positive lesions in these brain areas, respectively (Kempf et al., 2016).

Our phosphoproteomics approach further revealed that Ser16 phosphorylation of the microtubule-binding protein stathmin1 was decreased in the cortex, but increased in the olfactory bulb of $APP_{swe}/PS1_{\Delta E9}$ compared to wild-type mice (Kempf et al., 2016). Because stathmin1 phosphorylation has been shown to induce microtubule destruction and subsequent hyperphosphorylation of tau (**Figure 1**), it seems reasonable to suggest that stathmin1-mediated mechanisms may be associated with neurofibrillary lesions in the cortex, but not in the olfactory bulb. Moreover, phospho-14-3-3 proteins, which are directly involved in the phosphorylation of tau (**Figure 1**) *via* GSK3β, were markedly increased in the olfactory bulb over control levels, but remained unaltered in the cortex.

These data suggest that mechanisms and signalling pathways known to mediate the phosphorylation of tau *in vivo* are likely to induce neurofibrillary lesions in $APP_{swe}/PS1_{\Delta E9}$ mice in a brain region specific manner. To further illustrate the complexity of Mapt modulation, endogenous mouse tau has been previously shown to be differentially phosphorylated in post-synaptic density fractions *vs*. whole brain lysates of wild-

type animals, demonstrating a cellular compartment specific aspect of tau regulation in the brain (synapse *vs*. soma; Morris et al., 2015).

Conclusion and future perspective/application: Using mass spectrometry, bioinformatics and a double transgenic model of AD, we previously identified novel phosphosites on endogenous 'wild-type' mouse tau and other microtubule-regulating proteins. The observed phospho-proteome alterations were characterised by a brain area-specific signature and were associated with the presence of argyrophilic lesions in the olfactory bulb, cortex, hippocampus, but not the brainstem of $APP_{swe}/PS1_{\Delta E9}$ mice. These data demonstrate the utility of combining phospho-proteomics with *in situ* labelling techniques to link novel tau modifications to the development of NFTs in AD research.

Several important points can be made from the very limited number of studies that have assessed PTMs at physiological levels of mouse tau by using mass-spectrometry. Notably, studies of Kempf et al. (2016) and Morris et al. (2015) indicate differences in tau modification that are amyloidosis model-specific. Despite methodological differences in the workflow between the two studies, the fact that endogenous tau was modified in $APP_{swe}/PS1_{\Delta E9}$, but not J20 transgenic mice, invites further consideration of PS1´s role in tau regulation. From a technical point of view, it is clear to us that mass spectrometry should be run on both tau insoluble and soluble fractions, to enable a complete picture of the phospho-proteome contributing to the development of NFTs in the cell/brain tissue. Moreover, our efforts to characterise phosphosites on Mapt and other proteins should be quantitative rather than limited to basic event identification, as shown by Di Domenico et al. (2011) in the hippocampus of AD patients using 2D gels. Thus, it is necessary to normalise the quantitative data of the phospho-proteome against the proteome to ensure that the noted changes on phosphorylation levels are due to phosphorylation itself and not due to expression changes in the proteome, as originally proposed by the lab of Steven Gygi (Harvard Medical School, Boston, USA). This quantitative approach would be also advantageous in relation to the bioinformatics analysis of signalling pathways and pathway networks, which would be rendered more accurate/physiologically relevant.

Translating phospho-proteomics observations from murine models of familial AD to AD patient brains is warranted, albeit not without caveats. A key challenge relates to the long *post-mortem* interval times for obtaining human tissue, a delay which may itself induce secondary and tertiary processes in the (phospho-)proteomic profiles, obscuring changes due to the primary causes of AD (Brinkmalm et al., 2015). Furthermore, it is rather difficult to obtain brain biopsy samples from patients at the early stages of AD, and thus surrogate markers, such as those in the cerebrospinal fluid may need to be collected and studied. Another point to be addressed is the assessment of adequate sample size in order to reach meaningful statistical conclusions from experiments involving human material. Last but not least, there is a clear need to harmonise experimental protocols on an international level, particularly in the neuroproteomics community, so as to enable result comparisons and minimize technical variation. We believe that quantitative peptide labelling approaches such as TMT or iTRAQ together with an internal standard spiked in before processing the samples would improve comparison of performance between different laboratories.

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