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Microtubule affinity regulating kinase (MARK/Par1) isoforms differentially regulate Alzheimer-like TAU missorting and Aβ-mediated synapse pathology

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Importance of TAU protein for dementia syndromes: Dementia currently affects about 55 million people worldwide, with Alzheimer´s disease (AD) being the most prevalent form. The one crucial pathological hallmark of AD that correlates best with loss of synapses and cognitive decline are the so-called intracellular neurofibrillary tangles composed of mislocalized/missorted and hyperphosphorylated TAU protein (Naseri et al., 2019). Many other neurodegenerative diseases, both genetic and non-genetic, are characterized by neurofibrillary tangles or pathological accumulation of the protein TAU and are thus termed "tauopathies". Tauopathies include AD and related aging-associated dementia syndromes like frontotemporal dementia and variants thereof (progressive supranuclear palsy, Pick's disease, corticobasal degeneration), but also childhood-onset genetic diseases (Zimmer-Bensch and Zempel, 2021).

Importance of TAU missorting in disease: The protein TAU, encoded by the microtubule-associated protein tau (MAPT)-gene, is a neuronal protein physiologically mainly localized in the axon, regulating microtubule (MT) dynamics, and microtubule transport, synaptic function, and more (Morris et al., 2011). In AD and related tauopathies, TAU is mislocalized to the somatodendritic compartment ("TAU missorting"), which we have modeled in murine primary, human induced pluripotent stem cell-derived and SH-SY5Y neuroblastoma cell-derived neuronal models (Bell and Zempel, 2021, 2022; Bell et al., 2021). Our previous studies, as well as data from many other laboratories demonstrate that TAU missorting is crucial for TAU toxicity (Ittner and Ittner 2018; Zempel and Mandelkow, 2019). While several downstream mechanisms regarding how missorted/dendritic TAU may execute its synaptotoxic function (e.g. via cytoskeleton disruption via TTLL6/SPASTIN, or synaptic impairment via FYN/PSD95, etc.) are being discussed, upstream regulators of physiological TAU sorting and pathological missorting are less studied. Physiological TAU sorting may depend on mRNA-based (mRNA transport and compartment-specific translation) or protein-based mechanisms. The latter includes a TAU diffusion barrier located in the axon initial segment, and microtubule-based active transport/sorting into the axon. Both mechanisms fail when TAU is phosphorylated at the KxGS motifs present in all 4 repeat domains, which are in turn part of the MT-binding domain: TAU can pass the TAU diffusion barrier retrogradely and anterogradely when pseudophosphorylated at the KxGS motifs, and cannot bind microtubules anymore, which results in impaired microtubule-based trafficking/transport (Zempel and Mandelkow, 2019).

Molecular properties of TAU: TAU protein consists of 8 splice-isoforms, 6 of which are found in the human central nervous system. TAU contains up to 85 phosphorylation sites and thus is under the regulation of many kinases and phosphatases. One of these are microtubule affinity regulating kinases (MARKs) which phosphorylate TAU at an AD-specific Ser262 residue located within TAU´s MT-binding site on the second KxGS motif (Hanger et al., 2009). The somatic TAU accumulations and aggregations found in AD and related tauopathies brains show pathologically elevated phosphorylation at these sites (Augustinack et al., 2001). Physiologically, phosphorylation at the KxGS motifs leads to reduced TAU-microtubule interaction, resulting in decreased MT stabilizing function of TAU. The most important kinase (family) inducing KxGS phosphorylation of TAU, and thereby possibly the most important upstream regulator of TAU missorting, is MARK.

MARK, MARK regulation and impact on TAU: MARKs consist of four different isoforms encoded by different genes (MARK1, MARK2, MARK3, MARK4, with various splice isoforms each). They belong to the calcium/calmodulin-dependent protein kinase superfamily (Annadurai et al., 2017) and are activated through phosphorylation of a threonine residue in their catalytic domain by MARK kinase or liver kinase B1. In addition, at least MARK2 can be activated by phosphorylation in its kinase domain by calcium/calmodulin-dependent protein kinase I. MARKs are inhibited by phosphorylation of a serine residue near their threonine activation site by glycogen synthase kinase 3b, whereby the serine residue can no longer interact with other amino acids. MARK activity is also reduced by interaction, such as Par-5 binding to the catalytic domain or the spacer region. Furthermore, molecule folding (dimerization) can occur and lead to the autoinhibition of MARKs (Naz et al., 2013). Generally, MARKs play a key role in cell polarity establishment and maintenance. Furthermore, they are crucial for intracellular signaling, as well as protein stability and cell cycle control (Annadurai et al., 2017).

MARK-type TAU phosphorylation, physiologically taking place in growth cones and the somatodendric compartment, leads to somatodendric TAU-missorting in some, but not all experimental conditions (Zempel et al., 2013). Furthermore, MARK-type TAU phosphorylation results in cellular transport inhibition, impacts postsynaptic molecular makeup and can induce either spine enlargement or TAU toxicity as well as spine decay-depending on expression levels and duration (Zempel et al., 2013; Zempel and Mandelkow 2019).

Thus, MARKs and TAU may be crucial for proper axodendritic development, regulation of neuronal intracellular transport and the formation of synapses. Whether they have different physiological functions and roles in AD/tauopathy pathophysiology is unknown. Despite the likely importance of MARK in (TAU-based) AD pathology, the individual physiological roles of the MARK isoforms and their impact on TAU toxicity are understudied.

Perspective evidence pointing to differential effects of the different MARKs on TAU: We recently found that amyloid-beta (Aβ) toxicity is mediated by TAU, but only when TAU is phosphorylated or phosphorylatable at MARK-type phosphorylation sites, the KxGS motifs in the repeat domains of TAU. On the other hand, rat MARK2 transfection and transduction prevented oligomeric Aβ (oAβ) induced toxicity, i.e. loss of spines and missorting of TAU (Zempel et al., 2013).

We here present preliminary data indicating that the individual MARKs may differ in subcellular localization, may drive or prevent (post-)synapse formation, and may mediate or prevent TAU-missorting and toxicity. We found that the individual MARKs show very different subcellular localizing patterns, may drive or inhibit dendrite and spine formation, and may mediate Aβ toxicity to different extents – or prevent it. With this perspective, we aim to raise awareness for the need to differentially consider the individual MARK isoforms in future studies, which is currently largely neglected.

So far, no study has addressed isoform-specific sublocalization of MARKs in mammalian neuronal cells. Our preliminary data suggest very different localizations of each MARK-isoform leading to various, partly antagonistic effects on TAU. We transfected the 4 human MARK isoforms into mature rat primary hippocampal neurons, using methodology extensively described before (Zempel et al., 2017b). Cellular distribution differed remarkably: MARK1 was ubiquitously distributed, but MARK2 showed axonal exclusion and strong dendritic spine enrichment, MARK3 was mainly present in the soma and dendrites, MARK4 showed distinct enrichment in spines similarly to MARK2-isoforms, and was the only MARK enriched in the axon (**Figure 1A** and **D**). These data clearly show that the four MARKs differ significantly in their subcellular distribution, ranging from axonal enrichment (MARK4) to axonal exclusion (MARK2), and strong dendritic spine enrichment (MARK2/4) to absence in/of spines (MARK3).

Furthermore, these and similar experiments conducted in wildtype murine primary neurons (and Mapt-knockout (KO) mice, see below) demonstrated individual MARK-influence on cell morphology: MARK2 led to shortened, dense dendritic processes, as well as increased postsynaptic density. A similarly increased spine density was visible in cells transfected with MARK4. MARK3, on the other hand led to an absence of spines and changed axodendritic morphology as in increased dendrite lengths, while MARK1 did not have apparent effects (**Figure 1A–D**). TAU-phosphorylation at the KxGS-motifs after MARK1-4 transfection was analyzed in the same cells using the phosphorylation-dependent antibody 12E8. Phosphorylation levels, however, were only slightly elevated compared to untransfected cells (**Figure 1B**, **C**, **E**, and **F**). From our perspective, this could be based on little physiological interaction between the axonal TAU-protein and MARKs due to their localization patterns (with MARK1-3 not axonally enriched). In view of the axonally very present MARK4 however, other mechanisms (e.g. low axonal activation by upstream kinases of MARK or high axonal phosphatase activity) may be at play.

By overexpressing the different MARK isoforms in primary neurons derived from TAU/Mapt-KO mice or wildtype control mice, we also tested whether the effect of elongated dendrites in MARK overexpressing cells depends on the presence of TAU. We found that neurons from Mapt-KO mice have longer dendrites in control conditions (**Figure 1D**), hinting towards known compensatory upregulation of other MAPs implicated in axodendritic outgrowth (e.g. MAP1A, MAP1b, etc). Neurons derived from wild-type mice showed the same trend we observed in more mature primary neurons from rats: MARK2 expressing cells showed a decrease in maximal dendritic length and had shorter dendrites in general, while MARK3 expressing cells showed an increase in maximal dendritic length (**Figure 1A** and **D**). MARK1 and MARK4 expression had no effect in this regard. Interestingly, in neurons from Mapt-KO mice, expression of the different MARKs did not strongly impact the increased maximal dendritic lengths, although MARK2 expression may still result in shorter dendrites (**Figure 1D**).

Moreover, we observed that transfection of the different isoforms of human MARK (hMARK) changes the response of rat primary neurons to oAβ. OAβ is capable of inducing 12E8 recognizable TAU phosphorylation and activation of MARK in primary rodent neurons (Zempel et al., 2013). The number of spines was reduced in response to oAβ in cells transfected with MARK1,3,4, but not MARK2. Phosphorylation of TAU at the KxGS motifs was slightly enhanced in all cases, but particularly severe in the case of hMARK1 and hMARK3 (**Figure 1F** and **G** and data not shown). Thus, human MARK2 (similar to rat MARK2) (Zempel et al., 2013) protects against oAβ-mediated spine loss and downstream toxicity. The other MARK isoforms, particularly MARK3, may mediate oAβ toxicity at least in part via TAU phosphorylation at the KxGS motifs.

This possible mediation is underlined by the effects of MARK transfection on TAU phosphorylation and spine morphology being greater in Aβ treated cells than in

untreated cells in general (**Figure 1A**, **F**, **G** and data not shown). Aside from MARK-mediated Aβ induced TAU phosphorylation, these data could also point towards MARK-mediated TAU phosphorylation potentially requiring additional triggers. A potential mechanism underlying MARK-mediated TAU phosphorylation may be MARK activation and inactivation by various kinases (named above) at different sites. Aβ however, usually being targeted to dendritic spines, might induce spine-signaling that directly or indirectly alters the kinases affecting the activity of MARK. Another possibility might be MARKs being activated in response to Aβ-mediated changes in MT dynamics with TAU phosphorylation as a bystander effect. This might be either due to pathological changes of microtubules induced by Aβ (directly via changed calcium or SPASTIN activity, or indirectly via the loss of mitochondrial function physiologically providing energy for MT stability (Zempel et al., 2013; Tjiang and Zempel, 2022), or due to increased amounts of TAU present, as Aβ exposure induces TAU missorting into the somatodendritic compartment, where MARKs may be more active. The exact underlying mechanisms of these effects need further elucidation in future studies, in order to determine key players resulting in MARK activation in response to Aβ. This might reveal novel signaling pathways relevant for pathomechanisms of neurodegeneration.

Need for isoform specific studies and therapeutic strategies: In conclusion, all members of the MARK family have the potential to disrupt the microtubule binding of TAU, priming TAU for disease-associated and pathology-mediating TAU missorting. We have, however, previously shown that MARK activity may be essential for neuronal regeneration and re-establishment of TAU sorting. Here, we demonstrate that the different MARKs, while in principle highly homologous, show different subcellular localizations, effects on dendritic morphology, and the mediation of oAβ toxicity. Future studies aiming at therapeutic inhibition of MARK must pay careful attention to the MARK isoforms and disruption of MARK physiological functions.

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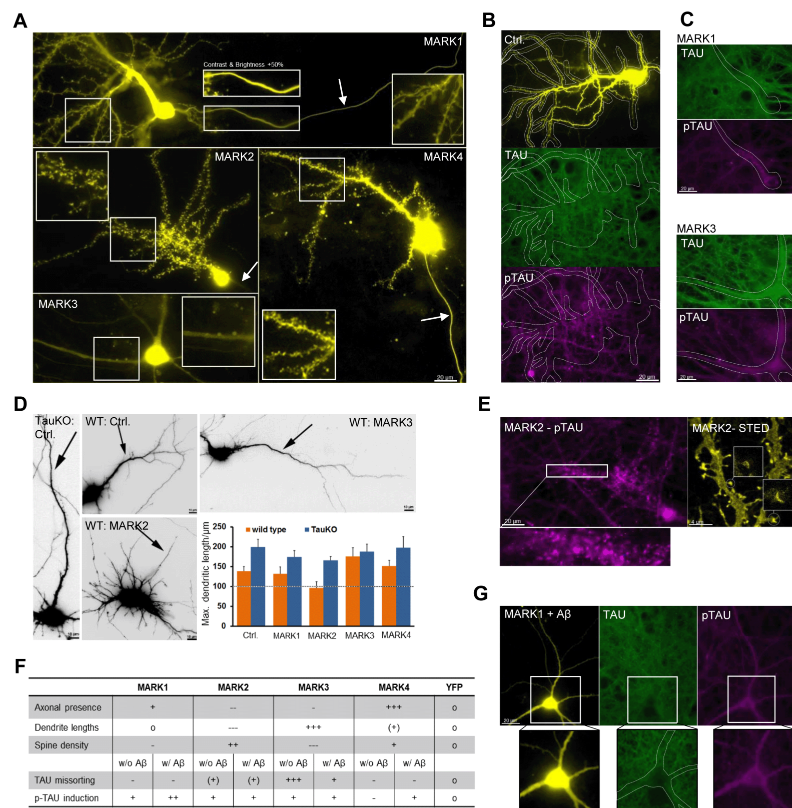

	MARK1		MARK2		MARK3		MARK4		YFP
Axonal presence	+		--		-		+++		o
Dendrite lengths	o		---		+++		(+)		o
Spine density	-		++		---		+		o
	w/o Aβ	w/ Aβ	w/o Aβ	w/ Aβ	w/o Aβ	w/ Aβ	w/o Aβ	w/ Aβ	
TAU missorting	-	-	(+)	(+)	+++	+	-	-	o
p-TAU induction	+	++	+	+	+	+	-	+	o

Figure 1 | Preliminary data show differential subcellular distribution and induction of spine formation, phosphorylation of TAU, and effects on dendrite outgrowth of the different isoforms of microtubule affinity regulating kinase (MARK).

Methodology as described in detail before (Zempel et al., 2017). (A–C, E, G) EYFP-tagged versions of MARK were expressed in primary rat neurons aged 21 days *in vitro* for 6 days. (A) Inserts show magnifications of boxed areas. Note the increased presence of MARK2 and MARK4 in spines, axonal exclusion of MARK2 but axonal enrichment of MARK4, short and dense dendritic processes of the cell transfected with MARK2, changed axodendritic morphology and absence of spines of the cells transfected with MARK3, and no apparent influence and ubiquitous distribution of MARK1. (B) Cells transfected with a control plasmid (EYFP) show no somatodendritic presence of TAU or phosphorylated TAU (KxGS motifs, 12E8 antibody). (C) Cells transfected with MARK1 show no increase in TAU missorting, and a marginal increase in 12E8 staining (upper panels), while cells transfected with MARK3 show increased somatodendritic TAU presence and slightly elevated 12E8 signals compared to control cells (B). (D) Different MARKs (and cotransfected tdTomato as a volume marker, shown here) were expressed in primary neurons aged 11 days *in vitro* for 5 days from wild-type or murine TAU/Mapt-knockout mice, the longest dendrite is marked by an arrow. Quantification reveals a TAU-dependent decrease of the longest dendrite in MARK2 expressing cells and an increase in MARK3 expressing cells. (E) Cells transfected with MARK2 show somatodendritic and dendrite-protrusion-localized presence of marked 12E8 signals. Confocal and stimulated emission depletion (STED)-superresolution (inserts) microscopy of MARK2 transfected cells show strong enrichment of MARK2 in spines and structures resembling post-synaptic densities. Circled areas are magnified in inserts. (F) Overview of the differential effects of MARKs, as observed in preliminary experiments in mouse and rat neurons transfected with the different isoforms of MARK and imaged for phospho-TAU (pTAU), TAU, and the MARKs as above (not all shown here). (G) Primary rat neurons aged 21 days *in vitro* expressing MARK1 for 6 days and treated with 1 µM of oligomeric amyloid-beta (oAβ) show increased phosphorylation of TAU at the KxGS motifs (by 12E8 staining) compared to control cells only transfected with EYFP (B) and treated with oAβ (not shown) or MARK1 transfected cells without oAβ treatment (C, upper panels). Unpublished data.

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