Tau: a phase in the crowd

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From the management of microtubules to the production of pathological species: liquid–liquid phase separation may tune the behavior of the protein tau in health and neurodegenerative disease. In this issue of The EMBO Journal, Hochmair *et al* (2022) demystify important aspects of tau condensate compilation.

On a daily basis, cells must orchestrate a plethora of routine processes as well as react to fluctuations in intracellular and extracellular signals. The biomolecules needed to execute these tasks are tightly packed in the confined intracellular space (Fulton, 1982). Cells create order in their crowded interior by compartmentalization in membrane-delineated and membraneless organelles (preprint: Keber *et al*, 2021). The latter are highly dynamic organizational units that emerge upon the de-mixing of biomolecules from the intracellular fluid into condensed—even more crowded—liquid "droplets," a process termed liquid–liquid phase separation (LLPS). Membraneless organelles play crucial roles in cellular physiology that we are only starting to understand, yet their formation comes at a risk, as proteins involved in neurodegenerative disease pathogenesis undergo LLPS and their high concentration within droplets may foster irreversible pathological aggregation (Zbinden *et al*, 2020).

One of these proteins is tau, an intrinsically disordered microtubule-associated protein that deposits in pathognomonic aggregates in patients with Alzheimer's disease (AD), frontotemporal lobar degeneration, and other neurodegenerative tauopathies. The progressive nature of these incurable disorders has been attributed to the intercellular propagation of aggregation-inducing tau "seeds" that wander through neural circuitries (Gibbons *et al*, 2019). Yet, the mechanisms causing tau to abandon its important job as axonal microtubule-bundler and instead jeopardize neural integrity by mislocalizing, aggregating, and spreading are incompletely understood (Fig 1A and B). In 2017, two laboratories independently showed the ability of full-length tau to undergo LLPS *in vitro* (Hernández-Vega *et al*, 2017; Zhang *et al*, 2017), sparking the idea that condensation plays a central role in tau physiology and its transition to pathology. Indeed, tau droplets form in cultured neurons (Wegmann *et al*, 2018) and facilitate microtubule polymerization (Hernández-Vega *et al*, 2017), but more gloomy outlooks include the "aging" of tau droplets to a nondynamic state, coinciding with the emergence of seed-competent aggregates (Zhang *et al*, 2017; Wegmann *et al*, 2018).

What drives the formation of these functional but risky tau droplets? *In vitro* data indicate that homotypic tau–tau droplets form via tau self-coacervation, a process inducible by inert molecular crowding agents such as polyethylene glycol (PEG) (Hernández-Vega *et al*, 2017; Wegmann *et al*, 2018). In addition, tau-attracting polyanions can initiate complex coacervation, giving rise to heterotypic (e.g., tau-RNA or tau-tubulin) droplets (Hernández-Vega *et al*, 2017; Zhang *et al*, 2017). Employing state-of-the-art optical techniques, Hochmair *et al* (2022) now untangle the relevance of these tau LLPS modes under conditions simulating the intracellular fluid.

As the intracellular milieu is both crowded and comprises a multitude of negatively charged biomolecules, the authors mixed tau with the crowding agent PEG, RNA, or both and followed the size distribution of droplets by dynamic light scattering (DLS) (Fig 1C). At ion levels, mimicking those in the intracellular fluid, tau-RNA droplets were unable to assemble. However, when PEG was added, heterotypic tau–RNA droplets did form and were larger, more numerous, and more resistant to an increase in ionic buffer strength compared to homotypic tau–tau droplets formed in the presence of PEG alone. Importantly, in a cell-free functional assay (Hernández-Vega *et al*, 2017), tau–RNA droplets nucleated microtubule bundle polymerization more efficiently than tau–tau droplets under crowded conditions (Fig 1D). Therefore, molecular crowding and RNA team up to generate robust and operational tau condensates.

Dynamic light scattering allows the detection of particles with a size well below the resolution limit of confocal microscopes commonly used to visualize droplets. This sensitive method revealed the presence of not only tau monomers (~ 10 nm) but also mesoscopic tau clusters (~ 50–150 nm) that spontaneously formed prior to the addition of PEG and/or RNA to the solution but vanished upon the formation of microscopic droplets (~ 1,000 nm). Although the lack of detection of the mesoscopic tau clusters in buffers with higher ionic strength warrants further examination of their physiological relevance, this observation is of high interest in the light of recent data pointing to a major contribution of mesoscopic liquid-like assemblies (~ 100 nm) to cytoplasmic organization (preprint: Keber *et al*, 2021). It is therefore important to determine whether mesoscopic tau assemblies also exist in the crowded interior of neurons. Perhaps, such clustering at the mesoscale brings tau in a ready-to-react state, allowing the rapid formation of visible tau droplets to execute on-demand cellular functions, such as microtubule nucleation.

Hochmair *et al* (2022) next questioned whether crowding and RNA also join forces during tau condensation in a pathological context. As expected (Wegmann *et al*,
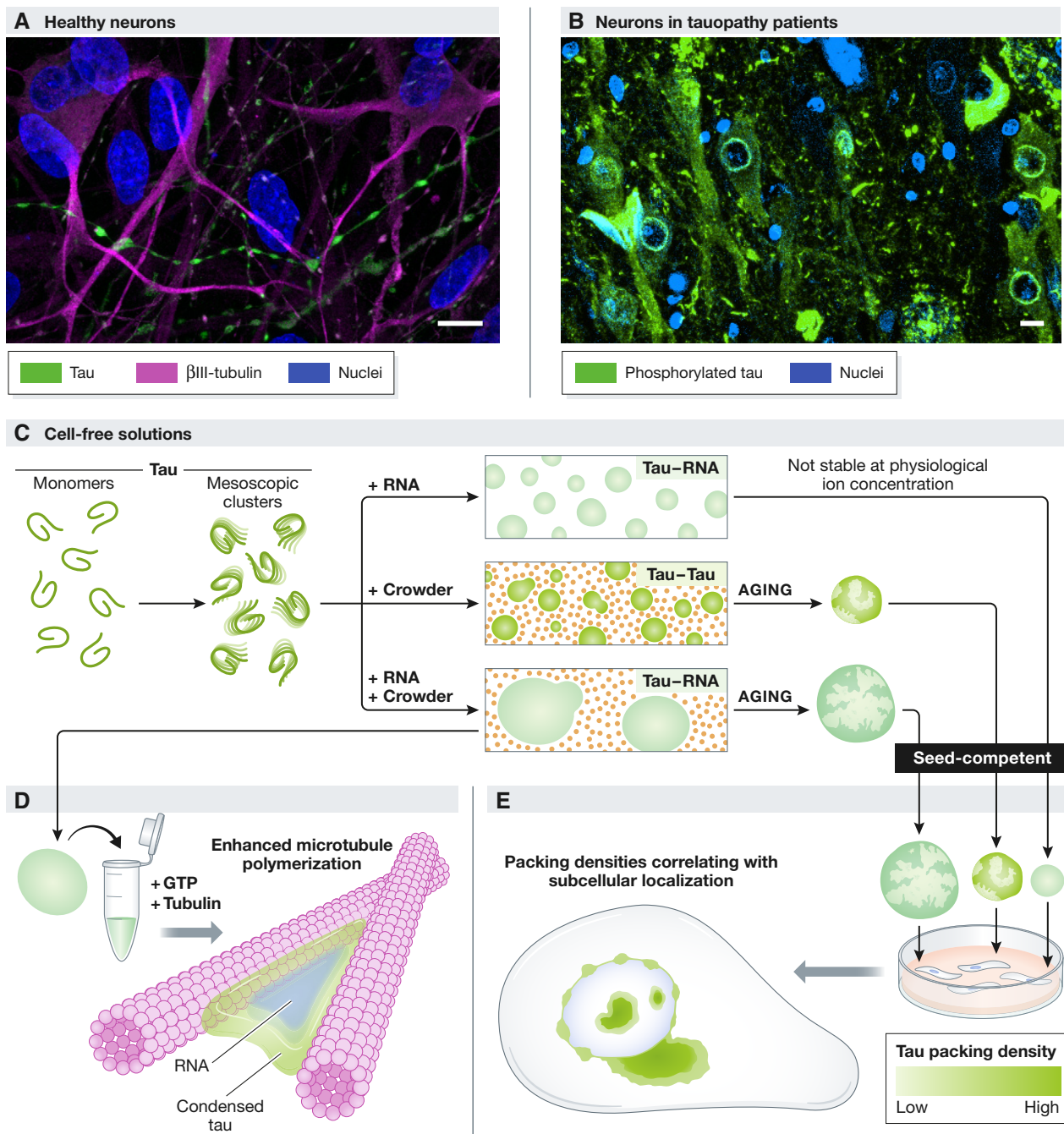
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Figure 1. Condensate properties affect physiological and pathological tau activity.

Tau associates with axonal microtubules in healthy neurons (A) but aggregates in the soma and at the nuclear membrane in tauopathy patients (B). Evidence is mounting that tau LLPS plays an important role in both processes. In cell-free solutions (C), tau monomers spontaneously assemble into mesoscopic clusters. Macromolecular crowding and/or RNA induce microscopic tau droplets with distinct physiological (microtubule bundling (D)) and pathological (aging) properties. All tau droplets can form seed-competent species and trigger intracellular tau aggregation that harbors variable packing densities correlating with subcellular localization (E) and recapitulates the neuropathological spectrum of tau inclusions (B). (A) Tau (green, Tau-5, Invitrogen, AHB0042) and βIII-tubulin (magenta, ProteinTech, 10068-1-AP) immunolabeling in human neural networks (preprint: Hruska-plochan *et al*, 2021). (B) phosphorylated tau (green, AT8, ThermoScientific, MN1020) immunolabeling in the hippocampus of an AD patient. DAPI-stained nuclei are shown in blue in (A–B). Bars: 10 μm.

2018), tau–tau droplets lost their dynamic character over time, as evidenced by minimal fluorescence recovery after photobleaching (FRAP) of labeled tau after a day in a PEG-packed environment. Tau–RNA coacervates similarly hardened in the presence of the crowding agent, yet they retained over 60% of their original mobility in a PEG-free solution. Would the diminished aging of these

tau-RNA droplets translate into a lower pathological potential? Perhaps unexpectedly, this was not the case: 24 h-old droplets of any type induced aggregation of naïve tau *in vitro* and in commonly used HEK293 tau biosensor cells. These data indicate that droplet maturation is not essential for the sprouting of seed-competent tau species. Therefore, tau condensates formed in the presence of crowding, RNA, or both pose an equal risk of generating tau pathology.

The role of LLPS in pathological tau transitions was further probed in the droplet-exposed tau biosensor cells. In addition to bright intranuclear and cytoplasmic aggregates, the intracellular tau pathology elicited by tau–tau and tau–RNA droplets included a fainter inclusion type that presented as a beaded ring around the nucleus and consistently overlapped with nuclear pore proteins. Employing elegant fluorescence lifetime imaging microscopy (FLIM) strategies, the molecular packing density in these peculiar nuclear envelope inclusions was found to be looser than in the core, but similar to the shell of intranuclear and cytoplasmic tau aggregates (Fig 1E). These data align with a recent study (Kang *et al*, 2021) and jointly suggest that nuclear envelope inclusions represent an early phase in the tau aggregation cascade. Importantly, an identical, relatively faint, perinuclear tau accumulation pattern is observed in neurons in the AD brain and classified as "pre-tangle" tau pathology, a stage inferred to precede the formation of characteristic endpoint neurofibrillary tangles (Bancher *et al*, 1993). Furthermore, a sensitive proximity ligation assay yielded labeling around and inside neuronal nuclei in brain areas free of classical tau pathology and in asymptomatic AD stages (Bengoa-Vergniory *et al*, 2021), indicating that tau multimerization at the nuclear envelope is among the earliest events in human pathology. Notably, like droplet exposure, also seeding with AD patient-derived brain homogenate elicited nuclear membrane inclusions in the tau biosensor cells. Whether these loosely packed perinuclear tau inclusions are condensates formed via LLPS—as speculated (Kang *et al*, 2021; Hochmair *et al*, 2022)—and whether their formation is a prerequisite to the development of larger cytoplasmic inclusions requires further investigation.

Taken together, Hochmair *et al* (2022) combine a flurry of optical techniques in cell-free and cellular systems to unravel novel aspects of physiological and pathological tau LLPS, including the functional alliance between molecular crowding and RNA, the spontaneous assembly of mesoscopic tau clusters and the evolution of seed-competent species in the absence of droplet aging. Advances in our understanding of the LLPS behavior of hazardous proteins include discoveries on proteins like TDP-43, FUS, and α-synuclein that constitute the hallmark inclusions in other neurodegenerative disorders (Zbinden *et al*, 2020), and, like tau, often present with a juxtanuclear localization. The latter may result from aberrant LLPS and faulty interactions with nuclear import receptors and/or nuclear pore proteins, leading to errors in nucleocytoplasmic trafficking, an emerging theme in neurodegeneration (Moore *et al*, 2020). Future work will disentangle how aberrant LLPS of neurotoxic proteins contributes to tipping the balance between coordination and chaos in the crowded cellular milieu.

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