A simple human cell model for TAU trafficking and tauopathy-related TAU pathology

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The microtubule (MT)-associated protein TAU is highly abundant in the axon of human brain neurons, where it binds to and stabilizes MT filaments. Thereby, TAU regulates the dynamic (dis)assembly of MT strands and is involved in a wide range of neuronal functions. In Alzheimer's disease (AD) and other tauopathies, TAU is missorted into the somatodendritic compartment. TAU missorting is accompanied by (or leads to) abnormal TAU phosphorylation, MT destabilization, and loss of dendritic spines and mitochondria, eventually resulting in TAU aggregation, neuronal dysfunction and cell death (Arendt et al., 2016). Strikingly, the mechanisms of TAU sorting, and the detrimental cascade upon its failure, are still not fully understood.

Which neuronal cell models are available for studying TAU sorting? Primary rodent neurons are often-used (Zempel and Mandelkow, 2017), but have considerable limitations, including the need for animals and species-specific TAU-intrinsic (e.g. different isoforms) and TAU-extrinsic differences (e.g. different interactomes). Recently, human-derived induced pluripotent stem cells (iPSCs)-derived and neural progenitor cells (NPCs)-derived neuronal models became relevant for mimicking human disease conditions *in vitro*, also for TAU trafficking (Sohn et al., 2019), and aggregation (Choi et al., 2014). Human iPSC- and NPC-derived neurons have many benefits, but the neuronal differentiation of these cells is complex, time- and resource-consuming, and often results in heterogeneous cultures with moderate differentiation efficiency. In contrast, SH-SY5Y neuroblastoma cells are human-derived, robust and cheap in maintenance, highly proliferative, and, in contrast to post-mitotic neurons, accessible to all forms of stable genetic manipulation, which includes the TAU-encoding *MAPT* gene (Bell and Zempel, 2021). SH-SY5Y cells can be differentiated into neuronal cells (SH-SY5Y-derived neurons) with various procedures.

In this perspective, we outline the suitability of these cells (i) to study the TAU-intrinsic regulation of TAU trafficking, and (ii) the impact of cellular interaction partners. Further, we discuss the limitations in comparison to other cell models for studying (i) TAU-induced postsynaptic spine loss, (ii) end-stage TAU pathology-related aggregation, (iii) neuronal subtype-specific susceptibility for TAU pathology, and (iv) tauopathies caused by traumatic axon injury (TAI).

General features of SH-SY5Y cells: SH-SY5Y neuroblastoma cells have been used for decades to study general principles of neurobiology and tauopathies, despite the fact that they harbour several chromosomal abnormalities, complex rearrangements, and a copy number gain of the *MAPT* locus on chromosome 17 (Bell and Zempel, 2021). The pathological relevance of abnormal *MAPT* overexpression remains unclear. While *Mapt*-knock out mice with roughly 1.5-fold human TAU overexpression show no abnormalities, an increase of TAU levels is correlated with faster disease progression in sporadic AD patients. Clinically, only very few cases with *MAPT* microduplications are described showing a large phenotypic variety with both neurodevelopmental and neurodegenerative disorders.

The differentiation of naïve SH-SY5Y cells into neuronal cells (SH-SY5Y-derived neurons) within one to two weeks is well-established with various substances, such as retinoic acid (RA), brain-derived neurotrophic factor (BDNF) or nerve growth factor (Kovalevich and Langford, 2013). SH-SY5Y-derived neurons show important features of mature neurons, including pronounced neuronal polarity, axonal outgrowth, the neuron-typical separation of axonal TAU and somatodendritic MT-associated protein 2, expression of neuronal maturation markers (like neuronal nuclei, synaptophysin or the synaptic vesicle protein SV2), and neuronal excitability (Bell and Zempel, 2021). Of note, the differentiation efficiency is highly variable and depends on the used procedure (see below).

In SH-SY5Y-derived neurons, all six major human brain isoforms are expressed. The reported isoform ratios differ from the adult human brain, as the 0N3R isoform is most abundant (Bell and Zempel, 2021). Nevertheless, the principle and synchronous expression of six TAU isoforms outclasses the situation in rodent cell culture models, where only four of the six human isoforms are expressed. IPSC-derived neuron cultures express all six isoforms, depending on the differentiation method, earliest after one month of cultivation, and also with a non-human brain-like ratio.

The phosphorylation state of TAU directly regulates its MT-binding affinity and is thought to play a role in the process of axonal enrichment (Arendt et al., 2016). In SH-SY5Y cells, several known residues are phosphorylated including the epitopes AT8 (S199, S202), AT180 (T231, S235), 12E8 (S262), and PHF1 (S396, S404). Many known TAU-related kinases (MAPK, CDC2, CDK5), phosphatases (PP1, PP2A) and likely other PTM-modifying enzymes regulate TAU in SH-SY5Y cells (Bell and Zempel, 2021). Thus, the presence of major TAU PTM-modifying enzymes in SH-SY5Y-derived neurons is plausible, which allows to study the interplay of TAU trafficking and PTM regulation, including isoform-dependent differences in the phosphorylation state and MT-binding affinity.

TAU-intrinsic factors involved in TAU trafficking: We recently discovered that SH-SY5Y-derived neurons sort endogenous TAU with a slightly lower efficiency than mouse primary neurons and iPSC-derived neurons. However, SH-SY5Y-derived neurons show and tolerate transient overexpression of transfected TAU much longer and achieve endogenous-like sorting efficiency, in contrast to primary rodent neurons (**Figure 1A–D**) (Bell et al., 2021). This enables us to study TAU trafficking of truncated, modified or otherwise engineered TAU constructs. Initial data with a truncated C-terminus-lacking TAU construct show similar sorting behaviour in SH-SY5Y-derived neurons, mouse primary neurons and iPSC-derived neurons (Bell et al., 2021). Hence, we consider a comprehensive analysis of domains, motifs, or interaction sites required for successful sorting feasible in SH-SY5Y-derived neurons.

Cellular and axon initial segment-specific factors involved in TAU trafficking: The axon initial segment (AIS), a highly specialized region at the proximal axon with ankyrin G (ANKG) as a master organizer (Rasband, 2010), is critical for developing neuronal polarity and action potential generation. In rodent primary neurons, ANKG or the tripartite motif-containing protein 46 (TRIM46) were critical for successful axonal TAU sorting (Rasband, 2010; Van Beuningen et al., 2015). Surprisingly, SH-SY5Y-derived neurons show efficient TAU sorting without any detectable accumulation of ANKG and TRIM46 at the proximal axon (**Figure 1E** and **F**). This lack of a classical AIS in SH-SY5Y-derived neurons could be the result of neuronal immaturity (primary rodent neurons develop TRIM46/ANKG accumulation at DIV3/4) and has to be considered as a potential limitation of mimicking the *in vivo* situation. However, this cell system bears potential for future studies, like addressing the importance of TRIM46-mediated MT polarization for TAU trafficking, and the general necessity of TRIM46 or ANKG for MT polarization at the AIS.

The (largely) ANKG/TRIM46-independent TAU sorting hints at the presence of unidentified mediators of neuronal (and TAU) polarity. Recent peroxidase- or biotinylation-based proximity labelling methods could be helpful to assess TAU-AIS interactions in SH-SY5Y-derived neurons, also in comparison with other neuronal cell models (Cho et

al., 2020). However, AIS-specific proximity labeling requires high sensitivity to detect transient interactions and a system enabling site-specific labelling without affecting the TAU trafficking process.

Synaptotoxicity and spine loss due to TAU missorting: Elevated levels of dendritic TAU result in mitochondrial mislocalization and postsynaptic spine loss via TAU-induced recruitment of the excitotoxicity-mediating kinase Fyn, or tubulin tyrosine ligase-like proteins that induce microtubule breakdown (Ittner and Ittner, 2018). The cascade from TAU missorting to spine degradation, however, is under debate. Suitable neuronal cell models must exhibit functional synapses and dendritic spine formation.

In SH-SY5Y-derived neurons, different pre- and postsynaptic markers as well as vesicle proteins are expressed (Bell and Zempel, 2021). However, the spatial distribution of these markers along axonal or dendritic processes does not faithfully recapitulate the *in vivo* situation. Robust co-localization of pre- and postsynaptic markers like seen in rodent primary neurons or in iPSC- and NPC-derived neurons is not observed. The obvious limitation of SH-SY5Y-derived neurons is the short lifespan of the cultures, with maximum growth periods of four to five weeks after RA/BDNF treatment. Thus, despite the reported excitability and the presence of functional synaptic vesicles in SH-SY5Y-derived neurons (Bell and Zempel, 2021), these cells might lack the degree of maturity that is necessary to mimic the synaptotoxic effects of pathological TAU in disease-burdened human neurons.

Tauopathy-related TAU aggregation: While many features of tauopathies like TAU missorting, hyperphosphorylation, or postsynaptic degradation can be induced in cell culture systems with external stressors, the formation of insoluble TAU aggregates is not inducible in most systems, including SH-SY5Y cells. Only with overexpression of pro-aggregant TAU mutants, TAU aggregates or inclusions can be obtained (**Figure 1A**, right panels). However, this artificial way of TAU aggregation, also performed in SH-SY5Y cells (Bell and Zempel, 2021), may generate aggregates reminiscent of certain genetic forms of tauopathy, but different from insoluble TAU aggregates found in AD brains. Strikingly, NPC-derived neurons were generated that successfully developed profound amyloid-β (Aβ) pathology, Aβ-induced TAU aggregation, and AD brain-like neuronal morphology after two to three months of cultivation (Choi et al., 2014). This AD-like pathology was achieved with lentiviral transduction of amyloid precursor protein and presenilin variants known from familial AD cases, and by using a three-dimensional Matrigel-based culture matrix. This indicates that NPC- or iPSC-derived neuron cultures are more promising for studying TAU aggregation, in principle due to the much higher culture life span, and their demonstrated ability to form aggregates composed of endogenous physiological TAU as seen in AD patients.

Neuronal subtype-specific susceptibility for TAU pathology: In many tauopathies including AD, the progression of TAU pathology is brain region-specific, e.g. the locus coeruleus is usually affected very early and even in asymptomatic individuals. This suggests that specific inter-neuronal differences are critical for the susceptibility to TAU pathology. However, the underlying neuron subtype-specific features remain enigmatic.

For SH-SY5Y-derived neurons, the reported neuronal identity include, depending on the differentiation treatment and the analysed biochemical markers, primarily noradrenergic, dopaminergic, or cholinergic neuronal subtypes (Kovalevich and Langford, 2013). Interestingly, these neuron types are found in subcortical nuclei that are early affected in many tauopathies including AD: the locus coeruleus, the nucleus basalis, and the substantia nigra pars compacta. Since the underlying pathomechanisms are still unclear, steerable generation of distinct SH-SY5Y-derived neuron subtypes would allow to study TAU-based toxicity in different neuronal subpopulations.

However, the reported identity of SH-SY5Y-derived neurons after specific differentiation procedures is inconsistent. Hence, the neuronal identities of SH-SY5Y-derived neurons might be not distinctive cellular subtypes, but rather accentuations of a spectrum of the same entity (Bell and Zempel, 2021). We conducted comparative analyses of four differentiation procedures but did not observe clearly distinct expression patterns of key enzymes commonly used to define neuronal subtypes (**Figure 1I** and **J**). Another principle obstacle is that general age-related changes in the cellular functionality, and major features of locus coeruleus, nucleus basalis or substantia nigra pars compacta neurons thought to have large impact on their increased susceptibility are certainly difficult to recapitulate in cell culture (Bell and Zempel, 2021).

Taken together, the resulting neuronal identities are ill-defined and might not recapitulate sufficient features of cells affected in AD and related tauopathies to be useful for studying neuronal subtype-specific susceptibility to TAU pathology.

Traumatic brain injury modelling with induced axon lesion: In one subgroup of tauopathies, including traumatic brain injury and chronic traumatic encephalopathy, mechanically evoked traumatic axon injury (TAI) precedes TAU pathology and NFT formation (Blennow et al., 2012). The underlying pathological cascade is still under debate. Although TAI mouse models exist, an *in vitro* laser-inducible axotomy cell model bears potential for several approaches. It allows the induction of precise lesions on a single-cell and even compartment-specific scale. Available live-cell imaging tools (e.g., photoconvertible TAU constructs, live AIS cytoskeleton markers and biosensors) would allow to monitor TAI-dependent alterations of TAU trafficking, phosphorylation, or the AIS architecture.

We tested whether SH-SY5Y-derived neurons are suitable for UV laser-induced axotomy. For this, we measured the somatic levels of transfected mTAU^{mCitrine} after axotomy, in comparison to uncut neurons (**Figure 1G** and **H**). Unfortunately, many neurons detached several hours after axotomy, impeding downstream analyses. Of note, preliminary experiments with primary mouse neurons and iPSC-derived neurons suggested a higher degree of attachment and viability (data not shown).

Although the experimental setup could still be improved to enable the use of SH-SY5Y-derived neurons, the data and experience suggest that alternative neuronal cell culture models are more suitable for laser-mediated axotomy.

Conclusion: SH-SY5Y-derived neurons are of human origin, capable of expressing all six human brain TAU isoforms, and exhibit many features of disease-relevant mature neurons. They are suitable for studying TAU sorting, as they show sorting of endogenous and transfected (physiological and truncated) TAU constructs similar to often-used neuronal cell models. The absence of classical AIS formation allows to study factors of TAU sorting that are independent of ANKG or TRIM46 enrichment.

In contrast, clear limitations of this cell model exist in comparison to other human and rodent neuronal cell models for studying (i) TAU missorting-induced spine loss, (ii) AD brain-like TAU aggregates, (iii) TAI-induced TAU pathology, and (iv) neuronal subtype-specific susceptibility to TAU pathology.

The present work was supported by the Else-Kröner-Fresenius Stiftung & Köln Fortune (both to HZ), and the Studienstiftung des deutschen Volkes (to MB).

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Date of submission: March 18, 2021
Date of decision: April 8, 2021
Date of acceptance: May 25, 2021
Date of web publication: August 30, 2021

https://doi.org/10.4103/1673-5374.322450
How to cite this article: *Bell M, Zempel H (2022)*

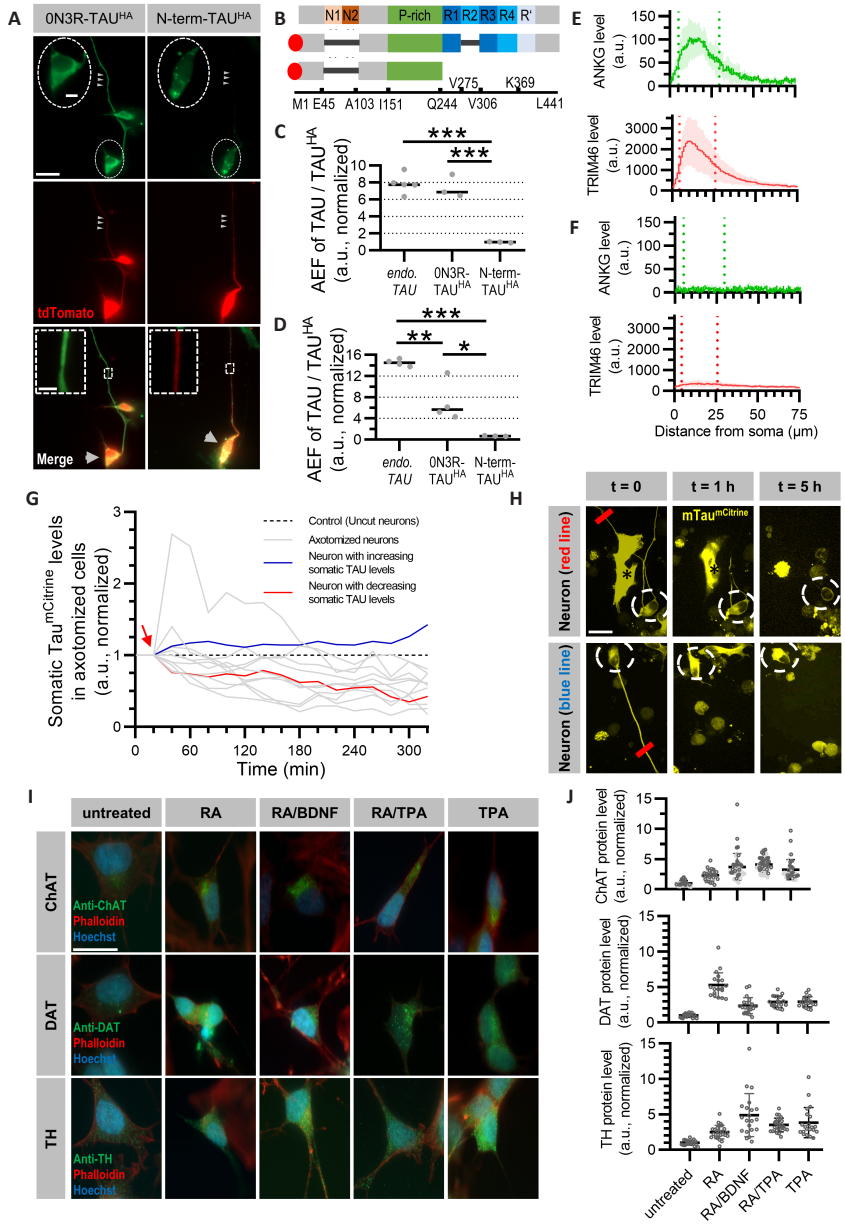

Figure 1 | **SH-SY5Y-derived neurons for studying TAU trafficking and tauopathy-related TAU pathology.**
(A) SH-SY5Y cells differentiated with RA/BDNF into neuron-like cells. Axonal targeting of transfected 0N3R-TAU and truncated N-term-TAU (lacks the C-terminal half, see B), both N-terminally fused to an HA tag, were determined, via normalization to co-transfected tdTomato as volume marker. Dashed circles and boxes show magnifications of the soma and axon, respectively. Scale bar: 20 µm, scale bar (dashed circle): 5 µm, scale bar (dashed box): 3 µm. (B) Illustration of the used TAU constructs compared to full-length 2N4R-TAU (top). The red ovals indicate the HA tag. N1, 2: N-terminal insert 1, 2; R1–4: repeat domains 1–4; R': pseudo-repeat domain. The bottom line shows relevant amino acid positions (one letter code + position within the human 2N4R-TAU sequence). (C, D) Quantification of axonal TAU enrichment of endogenous TAU and transfected TAU constructs in SH-SY5Y-derived neurons (C) and in mouse primary forebrain neurons (D). Significance levels: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (E, F) Protein levels of ANKG and TRIM46 at the proximal axon of SH-SY5Y-derived neurons (E) and mouse primary forebrain neurons (F). The dashed lines indicate the putative AIS region, i.e., the region where ANKG and TRIM46 signals are above 50% of the maximum value, determined for mouse primary neurons. (G, H) Somatic mTAU^{mCitrine} levels in SH-SY5Y-derived neurons after laser-induced axotomy, normalized to non-axotomized neurons. Cells were differentiated with RA/BDNF, transfected with mTAU^{mCitrine} at 5 days and axotomized at 14 days, followed by live-cell imaging for 5 hours. (G) All permanent lines symbolize single neurons ($n = 12$). Colored neurons are depicted for illustration in H. The mTAU^{mCitrine} signals were normalized against the average signal of uncut neurons ($n = 9$). The time point of axotomy is indicated (red arrow). (H) Time course of somatic mTAU^{mCitrine} signal from depicted neurons. The dashed circles comprise the analysed somata, the red bars indicate the site of axotomy. Asterisks indicate an adjacent non-neuronal SH-SY5Y cell expressing mTAU^{mCitrine}. Scale bar: 20 µm. (I) Expression of ChAT (key enzyme of cholinergic neurons), DA (dopaminergic neurons), and TH (catecholaminergic neurons) in differently differentiated SH-SY5Y-derived neurons as indicated. Scale bar: 20 µm. (J) Quantification of ChAT, DAT, and TH levels was done for one or two (untreated, RA/BDNF, RA/TPA, TPA in ChAT) independent experiments with 20–25 cells per experiment. Untreated SH-SY5Y cells are considered immature catecholaminergic, while differentiated cells are considered primarily noradrenergic (RA), dopaminergic (RA/TPA), cholinergic (TPA) or all of them (RA/BDNF). (A–F) Reprinted from Bell et al. (2021), Axonal TAU Sorting Requires the C-terminus of TAU but is Independent of ANKG and TRIM46 Enrichment at the AIS, pp 155–171, 2021, with permission from Elsevier. See original article for details and technical procedures. (G–J) Unpublished data. AEF: Axonal enrichment factor; AIS: axon initial segment; ANKG: ankyrin G; BDNF: brain-derived neurotrophic factor; ChAT: choline acetyltransferase; DA: dopamine transporter; HA: hemagglutinin; mTAU: mouse TAU; RA: retinoic acid; TH: tyrosine hydroxylase; TPA: 12-O-Tetradecanoylphorbol-13-acetate; TRIM46: tripartite-motif containing protein 46.

A simple human cell model for TAU trafficking and tauopathy-related TAU pathology. Neural Regen Res 17(4):770-772.
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C-Editors: Zhao M, Zhao LJ, Qiu Y; T-Editor: Jia Y