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Axodendritic sorting and pathological missorting of Tau is isoform specific and determined by axon initial segment architecture

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Running title: *Isoform-dependent (mis)sorting of Tau*

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Abstract

Subcellular mislocalization of the microtubule-associated protein Tau is a hallmark of Alzheimer disease (AD) and other tauopathies. Six Tau isoforms, differentiated by the presence or absence of a second repeat or of N-terminal inserts, exist in the human CNS but their physiological and pathological differences have long remained remain elusive. Here, we investigated the properties and distributions of human and rodent Tau isoforms in primary forebrain rodent neurons. We found that the Tau-Diffusion-Barrier (TDB), located within the Axon-Initial-Segment (AIS), controls retrograde (axon-to-soma) and anterograde (soma-to-axon) traffic of Tau**.** Tau isoforms without the N-terminal inserts were sorted efficiently into the axon. However, the longest isoform (2N4R-Tau) was partially retained in cell bodies and dendrites, where it accelerated spine and dendrite growth. The TDB (located within the AIS) was impaired when AIS components (AnkyrinG, EB1) were knocked down or when glycogen-synthase-kinase-3-β (GSK3β, an AD-associated kinase tethered to the AIS) was overexpressed. Using superresolution-nanoscopy and live-cell imaging, we observed that microtubules within the AIS appeared highly dynamic, a feature essential for the TDB. Pathomechanistically, amyloid-β insult caused cofilin activation, F-actin remodeling, and decreased microtubutule dynamics in the AIS. Concomitantly with these amyloid-β–induced disruptions, the AIS/TDB sorting function failed, causing AD-like Tau missorting. In summary, we provide evidence that the human and rodent Tau isoforms differ in axodendritic sorting and amyloid-β–induced missorting, and that the axodendritic distribution of Tau depends on AIS integrity.

Tau is one of the major microtubule-associated-proteins (MAPs) in neurons and promotes axonal microtubule (MT) assembly and stabilization (1,2). In the human CNS, Tau occurs in 6 isoforms, which are characterized by the presence or absence of the second repeat (3R or 4R-Tau), or the presence or absence of N-terminal inserts (0N, 1N, or 2N-Tau; see Fig.1A for details) (3,4). In adult humans, the two 2N isoforms (2N3R, 2N4R) are underrepresented, only 3-4% each, compared to 16.7% each if all 6 isoforms were equally expressed. In adult

mice there is no 3R isoform, but the three 4R isoforms are more evenly represented (varying between 20% and 40% each). During development, the isoform ratio of human Tau shifts from predominantly 3R Tau isoforms to a roughly even ratio of 3R and 4R (table 1) (4-7). Tau is generally considered an axonal protein in mature neurons (8), but in Alzheimer Disease (AD) and other tauopathies, missorting of Tau protein into the somatodendritic compartment is an early pathological event (9,10). In cell culture and mouse models of AD and Frontotemporal Lobar Degeneration with Tauopathy (FTLD-Tau), missorting of Tau coincides with synapse loss and functional impairments (11-13).

Abnormal splicing that only affects the isoform ratios of Tau without altering the amino acid sequence is sufficient to cause FTLD-Tau (4,14,15). Tauopathies can be classified depending on the isoform of Tau which appears in pathological paired helical filaments, neurofibrillary tangles or Thioflavin negative accumulations of Tau (16-18). Pick Disease is a 3R Tauopathy, Corticobasal Degeneration (CBD) and Progressive Supranuclear Palsy (PSP) are 4R Tauopathies, AD is a 3R+4R Tauopathy, and familial cases of FTLD-Tau with mutations of Tau can be either 3R or 4R or 3R+4R Tauopathies (18). Although Tau isoform distribution and function may be crucial for disease development, these aspects are understudied.

The mechanisms proposed for the polarized distribution of Tau in adult neurons include: i) preferential binding to MTs in the axon, ii) preferential degradation in the somatodendritic compartment (19), iii) axonal transport of Tau mRNA (20), iv) preferred protein translation in axons (21), and v) preferred retention by an axonal barrier (22). Since most protein synthesis takes place in the cell body the bulk of Tau must transit from the cell body to the axon through the Axon Initial Segment (AIS) which roughly coincides with a retrograde Tau barrier. The AIS is enriched with cytoskeletal scaffolding proteins AnkyrinG and βIV spectrin, which together cluster Na_v and K_v channels and link them to the actin cytoskeleton (23). Functionally, the AIS plays a role in action potential initiation and modulation, as well as neuronal polarity (24). Several studies ascribe the barrier function of the AIS to be based on lipids, membranes, F-actin or MTs, depending on the components studied (22,25-29). The internal structure of the AIS and its relationship to barrier functions has remained elusive since its discovery (30) but recent studies revealed a dense, fibrillar/globular submembranous coat containing the classical AIS proteins (e.g., AnkyrinG, βIV spectrin) which covers MT bundles (31).

The AIS and neuronal polarity are established early in development *in vivo* and can be mirrored *in vitro*. With the emergence of the axon, Tau becomes gradually enriched in the axon concurrent with AIS establishment (32,33). Similarly, Tau isoforms change from the fetal to the adult distribution, due to alternative splicing (table 1). Also, the initially high Tau phosphorylation decreases with neuronal maturation, but in neurodegeneration Tau becomes "hyperphosphorylated", concomitant with missorting and aggregation. A major kinases acting on Tau is GSK3β, which phosphorylates many of the Ser-Pro or Thr-Pro motifs, and is implicated in neurodegeneration (34). GSK3β is important for AIS function as it is an integral part of the AIS architecture, where it induces local tethering of β-catenin and AnkyrinG (35).

We recently discovered a barrier that blocks the retrograde passage of Tau from the axon into the soma and dendrites (termed Tau Diffusion Barrier (TDB), located at the AIS) (22). Here, we investigate the relationship between the TDB and the AIS, and their effects on different isoforms of Tau. We found that the TDB has different retention efficiencies for different isoforms of Tau. The TDB functions not only in a retrograde fashion but also anterogradely: It partially retains the largest isoform of Tau in the dendrites, where it enhances dendritic growth and spine formation. Knockdown of structural components of the AIS decreases the Tau barrier efficiency. The Tau and AIS kinase GSK3β induces missorting of endogenous

and exogenous Tau, but independently of Tau phosphorylation. STED nanoscopy and live-cell imaging show that MTs are major constituents of the TDB in the AIS, with unusual dynamics. Exposure of neurons to Amyloid-β oligomers results in impairment of the F-actin based filter in the AIS and decreased MT dynamics, resulting in failure of proper axodendritic Tau sorting.

Results

(a) The Tau Diffusion Barrier is isoform selective

Previous studies have shown that there is a retrograde diffusion barrier for Tau at the AIS that retards the back-diffusion of Tau from the axon once it has entered it (22). These results were obtained using a Tau fusion protein with the photoconvertible GFP-derivative Dendra2 (36), linked to the N-terminus of full-length Tau (Tau^{D2}). This allowed to observe the movement of axonal Tau after local UV irradiation. Phosphorylated forms of Tau^{D2} were able to penetrate the barrier to some extent. In the earlier study we focused on the Tau barrier with regard to full-length Tau (2N4R) only and its modification by phosphorylation. However, Tau contains several domains which may also modulate its functions.

In the present study we tested all six isoforms of Tau (see table 1 for comparisons and nomenclature of the isoforms, and Fig.1A for diagram). Transfected species of Tau^{D2} were distributed throughout the cell (Fig.1B1,1B5, green emission of unconverted Tau^{D2}). Then, ~15µm of the proximal axon (~50µm away from the cell body) were irradiated with UV-light, causing the Dendra2 to convert from green to red fluorescence. This was then imaged for up to 1h and signals were measured in the axon or the cell body (indicated by red rectangles and red triangles, respectively) (Fig.1B).

The reference values are represented by Dendra2 alone (barrier fully open, unrestricted diffusion from axon into cell body, Fig.1C, fluorescence (F)~100 in a.u.) and the 8-repeat construct of Tau (barrier fully closed, F~2.5 (background), almost no detectable diffusion into cell body). For the 4R isoforms the barrier is relatively tight (F~5-10, teal bars), for two of the 3R isoforms there is intermediate leakiness, (F~25, blue bars). For 2N4R-Tau mutants the leakiness is intermediate to high (F~25 to 70, red bars). This is particularly apparent for the 4KxGE mutant of 2N4R-Tau (bar 9), where the 4 KxGS motifs in the repeats (= target sites of kinase MARK) were pseudophosphorylated to KxGE, causing loss of MT affinity (37)).

Tau carrying mutations derived from FTLD or PSP also shows decreased MT affinity (38). As the TDB correlates with Tau-MT interaction, we tested the TDB for (i) 2N4R-Tau mutated at sites causing FTLD (pro-aggregant Tau with deletion of K280), (ii) a control version of this mutation that cannot aggregate due to the insertion of two prolines (anti-aggregant Tau ΔK280PP), and the mutant A152T of 2N4R-Tau implicated in PSP (39). All of these mutations increased the leakiness of the TDB for retrograde diffusion back into the cell soma (Fig.1C, red bars). The example of A152T-Tau is remarkable because a single point mutation outside the conventional MT-binding region (aa~200 to 370) can enhance the mobility of Tau in neurons. One exception to the above classification is 2N3R-Tau where the barrier is almost tight (as in 2N4R), indicating that the second N-terminal insert (aa 74-102, exon 3) contributes strongly to the functional retention by the Tau barrier, even though it lies outside the MT-binding domain. Some of these variations correlate with Tau's affinity for MTs, but not all, so that we conclude that MT-affinity alone is not sufficient to explain Tau retention in the axon.

The diffusion rates of the Tau species can be assessed by the fluorescence increase in the cell body (as shown above), but also by the decay in the photoconverted area, which reflects the diffusion of Tau both towards the cell body (retrogradely) and away (anterogradely towards the axon terminal) (Fig.1D,E). The reference values are set by Dendra2 alone (rapid decay because of fast diffusion, short half life of ~2 min) and 8R-Tau (very slow diffusion because of tight MT binding, long half life of ~46 min). Overall, tight MT binding correlates with slow diffusion

within the axon (e.g. half times Dendra2<4KXGE-Tau <2N4R-Tau<8-repeat-Tau).

(b) Exogenous Tau is efficiently sorted into axons of wildtype and TauKO neurons except the largest isoform 2N4R

In the experiments described so far we overexpressed the isoforms of Tau^{D2} at high levels (~20 fold of endogenous Tau) to be able to investigate the barrier. In this setting, using primary neurons at an early stage of polarization (8-9DIV), Tau is initially present in all compartments. Subsequently, we photoconverted a subset of molecules in the axon to observe their propagation. As an alternative approach, to confirm the functioning of the TDB at low Tau levels, we used a protocol that limits Tau expression to 1-3 fold of endogenous Tau (estimated at ~1µM, (40)), which required antibody-mediated amplification of the transfected constructs. We also waited longer (4-6d) to allow the transfected versions of Tau to reach their destiny compartment. We also used more mature well-polarized neurons (13DIV) with longer processes from TauKO and wildtype mice. We found that the shorter human 4R isoforms (0N4R and 1N4R) and the 3R isoforms were highly enriched in axons, whereas the longest isoform (2N4R) was in part excluded from axons (Fig.2A,B, Fig.S1,2, table S1).

To quantify effects, we used an enrichment factor (e.f.), which is calculated by the normalized ratio of axonal presence vs. dendritic presence of transfected Tau (see methods). Quantitatively, axonal e.f. was ~75, 85, 95 for 2N4R, 1N4R, 0N4R, respectively. The 3R versions of Tau without or two inserts (i.e., 0N3R and 2N3R) showed axonal enrichment but to different extents (e.f. of ~90, 95), while the 1N3R isoform was with an e.f. of 70 less efficiently sorted (table S1, Fig.2C). With the exception of 2N4R-Tau^{D2}, Tau isoforms and mutants which were able to partially penetrate the TDB retrogradely were also axonally enriched in neurons transfected with a specific isoform. For example: 4KxGE-2N4R-Tau^{D2} showed retrograde penetration of the TDB (Fig.1B,C and (22)) and also axonal enrichment (i.e. anterograde penetration of the TDB), in contrast to other mutations (ΔK280, ΔK280PP, A152T, all 2N4R-Tau^{D2}) which showed only slight retrograde penetration of the TDB and correspondingly only limited axonal sorting (Fig.2A-C, S1,S2 and table S1). Since 2N4R-Tau has a much higher affinity to MTs than the tested mutations, this confirms that strong MT binding can only partly explain axonal sorting of Tau. Surprisingly we found no differences for TauKO (KO of all isoforms (41)) and wildtype cells (for full comparisons see Fig.S1,2), indicating that even when Tau is absent during development and after establishment of polarity, Tau can be as efficiently sorted as in wildtype conditions.

(c) Dendritic Tau accelerates spine formation and dendritic elongation

In rodents, 3R-Tau is mainly expressed during the 1st week of neuronal development and decreases during the 2nd week, while 4R-Tau is mainly expressed after two weeks. Because mice overexpressing the 2N4R isoform of Tau show enhanced LTP early in life (42) and throughout life (43), we hypothesized that Tau has a dendritic function in synapse formation. As dendritic synapses sit on spines, we chose to express the Tau isoforms in young neurons, before spines are formed (7DIV). We found that some isoforms of Tau^{D2} induce accelerated spine formation, but with strikingly different phenotypes (Fig.2D). For example, 0N3R induced elongated spine necks and mature spine heads, unlike filopodia (Fig.2D2). 0N4R had almost no effect, while 2N4R-Tau induced mature spine heads (Fig.2D, table S1). Expression of the other native isoforms of Tau showed intermediate effects on spine development, but all of them induced an increase in spine number and either an increase in spine maturity or spine length. In detail, 1N4R-Tau showed intermediate effect on spine maturation, number and length, positioning it right between 0N4R and 2N4R-Tau (table S1). 2N3R-Tau showed intermediate and 1N3R-Tau showed slight increase in all parameters investigated (spine number, length, maturity), positioning them

between 0N4R and 2N4R. The mutations tested (ΔK280, ΔK280PP, A152T, KxGE, KxGA; expressed on the basis of 2N4R-Tau^{D2}) resulted in more spines of variable phenotype, indicating that these mutations might affect the spine maturation and also spine/filopodia dynamics (Fig.2D, table S1). As overexpression of Tau results in enhanced spine maturation, we tested if there was an effect of acute knockdown of Tau via shRNA on spine maturation. When applied during the period of spine maturation (14-21DIV, which coincides with the transition from 3R to 4R Tau), knockdown of all Tau isoforms via shRNA results in impaired spine formation (consistent with (44)) (Fig.2D7,8). Yet, we found no differences between wildtype and TauKO neurons in terms of aberrant spine formation after transfection of individual Tau isoforms. This indicates that forced expression of different isoforms (thereby also neglecting the physiological mixture of isoforms) results in aberrant spine development independent of the presence or absence of endogenous Tau. Not surprisingly, expression of Tau induced elongation of dendrites, but to different extents (Fig.2E, Fig.2E,D graphically summarized in Fig.2F). Elongation of dendrites required the presence of N-terminal inserts and presence of intact KxGS motifs, but were largely unaltered in case of the mutation tested (ΔK280, ΔK280PP, A152T; all expressed on the basis of 2N4R-Tau^{D2}). Thus, elongation of dendrites correlates roughly with increased dendritic presence for some of the native Tau isoforms (high dendritic presence for N-containing isoforms results in elongated dendrites, while 0N-Tau isoforms do not elongate dendrites), but not for the Tau mutations tested and the 2N3R-isoform (intermediate dendritic presence). This indicates that presence of the two N-terminal inserts is sufficient to induce dendrite elongation.

(d) Structural components of the Axon Initial Segment are required for tightness of the anterograde and retrograde Tau Diffusion Barrier

Since the position of the TDB coincides roughly with that of the AIS we determined which of the major known components of the AIS are essential for TDB function. Interestingly, knockdown of any of the AIS components by shRNA, such as neuronal cell adhesion molecules (NrCAM), voltage-gated sodium channels (Na_v) or scaffold proteins (βIV spectrin or AnkG) (45)), lead to slight leakage of 2N4R-Tau^{D2} from the axon into the soma (4-6x above background levels (Fig.S3A,B)). This leakage is similar to the leakiness of one of the shorter isoforms of Tau (e.g. 0N3R, Fig.1C). In addition, knockdown of the MT end binding proteins EB1 and EB3, which are enriched at the AIS (46), lead to similar enhanced retrograde leakage of Tau^{D2} (Fig.S3A,B). Thus, each of these AIS components contribute to the maintenance of the retrograde TDB.

We next tested whether knockdown of the different structural components of the AIS would also lead to enhanced anterograde movement of Tau. We thus expressed the longest isoform of Tau (2N4R-Tau^{D2}), which shows little anterograde axonal propagation compared to the other isoforms (Fig.2A,B and table S1), in combination with knockdown of the AIS components (i.e. NrCAM, NaV, βIV spectrin, AnkG, EB1 and EB3; Fig.S3C,D, see Fig.S4A,D,E for controls). In control conditions (empty shRNA vector, unrelated protein (Spast)), 2N4R-Tau^{D2} is present slightly more in axons (~100µm from the cell body) than in the cell body or dendrites as shown above, and MAP2 signals are low in axons (~3-fold lower than in the cell body and 5-fold lower than in dendrites, Fig.S3C,D). Knockdown of the AIS components AnkG and EB1 results in an increase of 2N4R-Tau^{D2} in the axon by ~50%, while the other components (NrCAM, Na_v, βIV spectrin, EB3) show no measurable effect. Knockdown of any component of the AIS caused invasion of MAP2 into the axon (Fig.S3D). Interestingly, none of the knockdowns resulted in impaired axonal sorting of endogenous rat Tau (Fig.S4). Together these results indicate that the AIS structural components are important for both the regulation of anterograde and retrograde passage of exogenous Tau, but do not hinder anterograde passage of endogenous Tau.

The protein kinase GSK3β is a structural component tethered to the AIS, affects the activity of AIS-based Na channels (35) and is crucial for cell polarity and pathological development of tauopathies including Alzheimer's (47,48). We thus tested the effect of GSK3β on Tau distribution. Overexpression of GSK3β induces pronounced missorting even of endogenous Tau, in contrast to other structural components of the AIS mentioned above. Missorted Tau is phosphorylated at the KxGS-motifs (Fig.S5A), despite the fact that GSK3β cannot phosphorylate Tau directly at these motifs. This is consistent with the hypothesis that Tau becomes phosphorylated after it has transited into the somatodendritic compartment by other kinases already present there, e.g. MARK (49). Missorting of Tau is recognized as a pathological sign and can be induced by exposure to Aβ (see e.g. Fig.S5A1 arrowheads) and other forms of molecular stress (49). However, exposing GSK3β transfected neurons to Aβ did not further increase phosphorylation or missorting of Tau (Fig.S5A). This indicates that Aβ exposure and GSK3β activity either activate the same pathway, or GSK3β activity changes the cell in a different fashion so that Aβ cannot exert its toxicity anymore. We also tested the state of GSK3β activity after exposure of primary neurons to Aβ, using phosphorylation at S9 as a readout. Time slicing up to 24h showed that there is a brief 30min period of phosphorylation of GSK3β (inactivation), which then returns to baseline within 3h (Fig.S5B). This indicates that there is no activation of GSK3β after exposure to Aβ.

We previously found that Aβ-induced Tau missorting requires loss of dendritic MTs (12). Here we find that missorting of Tau is induced by overexpression of GSK3β (wildtype and constitutively active GSK3β-S9A), but not by inactive GSK3β-S9E, and thereby induced missorting of Tau does not lead to MT breakdown (Fig.S6A). Hence, MT loss cannot account for Tau missorting induced by GSK3β. We thus tested whether missorting of Tau induced by GSK3β is due to a change of Tau or a change of the TDB. Cotransfection of 2N4R-Tau^{D2} and GSK3β results in enhanced retrograde propagation of axonal Tau^{D2} into the cell body, indicative of a dysfunctional barrier, while inactive GSK3β (S9E mutant) has no effect (Fig.3A,B). GSK3β can phosphorylate Tau at many sites, possibly changing Tau's diffusion capability or interaction with the TDB. To determine the effect of GSK3β on the TDB independently of Tau phosphorylation, we cotransfected GSK3β with non-phosphorylatable Tau^{D2} (17AP-Tau^{D2}). 17AP-Tau^{D2} contains 17 proline directed serines (putative GSK3β phosphorylation sites) mutated to alanine (17AP-Tau^{D2}). 17AP-Tau^{D2} still results in enhanced retrograde propagation to the soma when cotransfected with GSK3β, but not when expressed alone or co-expressed with a control (CFP; Fig.3A,B)). Diffusion rates within axons are the same for 2N4R-Tau^{D2} irrespective of GSK3β activity (Fig.3C). However, 17AP-Tau^{D2} shows reduced diffusion by 50% already after 10min within the axon (Fig.3C, purple). This points towards an underestimation of the TDB dysfunction since there is less Tau available to cross the TDB. Taken together, this indicates that GSK3β influences Tau sorting via modifying either the TDB or some other factors maintaining the polarized distribution of Tau (e.g. inhibiting the Tau kinase MARK), but not by phosphorylating Tau.

(e) AIS structure contributes to Tau Diffusion barrier

Sequence motifs in the 5' or 3' UTR's of endogenous Tau mRNA have been implicated in the polarized trafficking and translation of Tau (20,21). Our exogenously expressed Tau lacks these motifs, yet the protein is also sorted in a polarized fashion. This suggests that the mRNA-based mechanisms cannot account alone for the sorting of Tau protein. Since known structural elements of the AIS are necessary to maintain the tightness of the Tau diffusion barrier (Fig.S3) we hypothesized that there may be additional structural elements of the TDB not integrated into the AIS structure that determine the isoform specific blockage of Tau. We previously showed that the efficiency of the TDB is decreased when MT dynamics are disturbed

by the MT destabilizer nocodazole, i.e. loss of MTs made the barrier leaky (22).

To examine MT-related properties the beginning of the AIS was defined as the point where an AIS marker (NrCam) reaches 50% of its level within the AIS, typically 17 µm away from the edge of the cell body. This coincides roughly with the point where MAP2 (a somatodendritic marker) decreases to ~50% of its value in the axon hillock (Fig.4A,B). Acetylated tubulin (a marker for stable MTs) starts low and increases slightly within the AIS, polyglutamylated tubulin (stable MTs and a potential target for MT-severing enzymes) remains low throughout the AIS (Fig.4A,C,S7). Tyrosinated tubulin (a marker for dynamic MTs) continuously decreases from the beginning of the AIS onward to the axon, consistent with mainly dynamic MTs in the cell body and mainly stable MTs in the axon (Fig.4C, for volume-normalized calculations and pictograms see Fig.S7). We next used fixation protocols that either facilitate the visualization of polymerized tubulin (MTs) or free tubulin. This revealed high levels of tubulin in the AIS compared to the axon or the cell soma (~2.5 fold higher), but only a low fraction of stainable MTs (Fig.4D). The ratio of free tubulin vs. MTs is ~2:1 in the AIS, ~1.5:1 in the cell body, but only 1:1 in the axon (Fig.4D). This indicates a predominance of unstable MTs or depolymerized tubulin in the AIS.

We next attempted to visualize MTs in the AIS with STED nanoscopy. Within the AIS, no MTs are observed when using monoclonal antibodies against unmodified α-tubulin (Fig.4E) or acetylated tubulin, and very few MTs are visible with an antibody against tyrosinated tubulin (Fig.4F) with normal fixation procedures (i.e. no application of MT modifying substances or permeabilization prior to fixation). This indicates that MTs within the AIS are too dynamic to be visualized with standard staining techniques. Only coapplication of the molecular densifier PEG (polyethyleneglycol), which creates high osmotic pressure allowing the fixatives to fix MT before they fall apart, together with the MT-stabilizer taxol prior to fixation, allowed us to observe MTs within the AIS, emphasizing the transient nature of these MTs (Fig.4G).

Next we examined MT dynamics directly by imaging of transfected EB3-GFP within the AIS. Due to the high density of dynamic MTs in the AIS (compared to the low density of dynamic MTs in axons, which are easier to image) a specialized imaging procedure was required to visualize EB3 comets within the dendrites and the AIS, using high frame-rate imaging combined with noise reduction (see methods). This revealed that within the AIS there is a high concentration of transfected EB3 and a high density of EB3-comets, compared to axons and dendrites (Fig.5A,B). This again illustrates that MTs are highly dynamic and short lived within the AIS. All comets observed within the AIS moved anterogradely, indicating a homogeneous polarity of MTs with plus ends pointing into the axon (in contrast to dendrites that contain MTs with mixed orientations (50)). The high concentration of EB3-comets hints to a hub or organization center for MTs at the AIS, likely affecting MT based transport. Costaining of EB3 in fixed cells with MAP2 and mitochondria illustrates that the EB3 hub lies within the AIS (Fig. 5C,D).

(f) Exposure of neurons to Aβ results in activation of cofilin, loss of F-actin, and decreased MT growth within the AIS

Missorting of Tau induced by Aβ oligomers is not caused by breakdown of the retrograde barrier, but by failure of newly synthesized Tau to sort into the axon (49). This could be due to either a tightening of the anterograde barrier, or loss of sorting-enabling structures. We thus tested if Aβ exposure connects pathological missorting of Tau with defective elements of the AIS/TDB.

We first tested whether Aβ binds to the AIS. Aβ localizes to dendrites (primarily dendritic spines), but less to axons (90% less binding, judged by immunofluorescence). In contrast, Aβ localizes fairly well to the AIS (25% of dendritic localization, Fig.S8A). As a result of Aβ exposure, AnkyrinG throughout the AIS and F-actin within the AIS decrease, indicative of a direct effect of Aβ on the AIS (Fig.S8B).

An F-actin meshwork represents a cytoplasmic barrier, which hinders non-axonal proteins and larger beads to enter the axon (26,27,51). We previously found that in dendritic spines, Aβ exposure leads to a rapid loss of F-actin (49). Testing F-actin content in the AIS both by live-cell imaging and phalloidin-based stainings, reveals that F-actin is lost and remodeled rapidly after exposure to Aβ, i.e. within 15 min, but not for example in the dendritic shaft, where F-actin accumulates (Fig.6A,B). As cause for this actin remodeling, we found that cofilin, a F-actin severing enzyme, becomes activated (dephosphorylated) in the AIS with a time course comparable to that of decreasing F-actin (Fig.6). Thus, activation of cofilin could result in loss or remodeling of F-actin. Live imaging with an F-actin sensitive probe (f-tractin) confirmed that the area covered by F-actin within the AIS increased, but only after 1.5h of treatment with Aβ (Fig.7A), while in control conditions there was no change. This timing is in agreement with the increase in missorting of Tau (49), and the decrease of cofilin activity after initial activation by Aβ (Fig.6). We next tested whether loss of F-actin alone would lead to missorting of Tau, due to simple loss of its cytoplasmic filter function. Treatment of primary neurons with various concentrations (0.5-2.5μM) of the F-actin destabilizing drug LatrunculinA (LatA) for various periods (5min-24h) did in no case lead to missorting of Tau, despite >90% reduction of F-actin staining upon treatment. Instead, co-treatment of primary neurons with LatA and Aβ led to an *attenuation* (50%) of Aβ induced missorting. Conversely, treatment of cells with the F-actin overstabilizer Jasplakinolide (1μM) was sufficient to induce missorting of Tau, even independently of Aβ (Fig.7C).

Thus, only after cell stress ceases, cofilin activity is attenuated and actin in the AIS repolymerizes. Tau entry into the axon is then blocked by aberrant repolymerization of F-actin in the AIS. This finding would be in agreement with earlier data which indicate that Aβ-oligomers lose their toxicity after a brief time (1-3h) in cell culture, and that the peak of missorting of Tau also appears only after the Aβ-oligomers lose their toxicity (49,52). How can the aberrantly polymerized F-actin block Tau entry? EB3-live imaging of the AIS revealed that 1.5h after Aβ insult, the EB3 comet speed was reduced by ~20% (0.1μm/s) (Fig.7B). This indicates that repolymerizing actin might exert its effect on Tau sorting via strangulating MT-polymerization within the AIS.

(g) Aβ exposure disrupts Tau isoform specific axodendritic sorting

Previously, we showed that Aβ causes impaired translocation of Tau into axons rather than retrograde leakage of Tau from the axon to the soma (49). The effect of Aβ on the TDB has not been determined so far. We transfected neurons with 2N4R-Tau^{D2} (strong dendritic presence) as well as 0N4R-Tau^{D2} (strong axonal presence) and measured axonal sorting in the presence and absence of 1μM Aβ. Axonal translocation was determined by photoconversion of Tau^{D2} in the soma and imaging of axon. 2N4R-Tau^{D2} is poorly sorted into the axon while 0N4R-Tau^{D2} is translocated to the axon and past the AIS (100 μm from the axon hillock) within 10 min. Aβ treatment decreases the axonal sorting of 0N4R-Tau^{D2} (Fig.7D). Conversely, 2N4R-Tau^{D2} and Dendra2 show increased axonal penetration after Aβ treatment (Fig.7D2). This hints towards an alteration of the AIS structure as a result of Aβ, likely related to the restructuration of F-actin in the AIS.

To test whether the sorting of rodent (mouse) Tau (mTau) isoforms is different from the human isoforms, we cloned the four rodent Tau isoforms (0N3R-mTau, 0N4R-mTau, 1N4R-mTau, 2N4R-mTau) tagged with Citrine (mTau^{Cit}). We found that mTau isoforms behave slightly differently from human Tau isoforms: Differences in axodendritic sorting among the isoforms are less pronounced, and only 0N4R-mTau^{Cit} is more sorted into the axon (axonal e.f. of ~100, vs ~85 of the other isoforms (Fig.7E)). This is in contrast to the human Tau isoforms, which show a wider variation between the isoforms (e.f. ranging from ~70 to ~95). We next tested the contribution of the isoforms to missorting by transfecting individual isoforms into

neurons, and then exposed cells to Aβ. This revealed that the axonal sorting of 0N4R-mTau^{Cit} is reduced (~30%) after Aβ insult, while the other rodent isoforms (0N3R, 1N4R, 2N4R) show no change (Fig.7E). In summary, we argue that missorting of Tau is due to somatodendritic accumulation of the normally axonally targeted 0N4R-Tau. Aβ-caused missorting can be induced at developmental stages where only 0NxR isoforms are present, but 0N3R-Tau distribution is unchanged after Aβ insult. Thus missorted Tau must consist mainly of 0N4R-Tau.

Discussion

a) The Tau Diffusion Barrier functions both anterogradely and retrogradely, and its tightness is isoform specific

In the present study we investigated the Tau Diffusion Barrier (TDB) and the function of the AIS with respect to physiological sorting and pathological missorting of Tau. We find that longer isoforms of Tau, 2N4R and 1N4R, show almost no retrograde passage from the axon into the soma, while all the shorter splice variants of Tau are able to overcome the retrograde barrier (Fig.1). The same short isoforms of Tau also show enhanced axonal enrichment (i.e. anterograde sorting) within 4-6d when expressed at near-endogenous levels (Fig.2). This finding was confirmed by photoconversion of Dendra2-tagged Tau in the soma, which did propagate slower into the axon in case of the longest Tau isoform (2N4R) compared to a medium sized, well axonally sorted Tau (0N4R; Fig.7D). This indicates that the TDB can function in both directions. Interestingly, isoform specific sorting of Tau occurs both in TauKO and in wildtype neurons, even when exogenous Tau is transfected after establishment of polarity. Thus the Tau sorting machinery is either not Tau specific and develops normally in the absence of Tau, or the cell can rapidly establish Tau sorting even after the process of neuronal polarization has been accomplished.

The isoform specific differences of anterograde sorting (i.e. axonal enrichment of somatically produced Tau) or retrograde passage from the axon to the soma are independent of MT affinity (53) or axonal diffusion rates (Fig.1,2). In case of 3R-Tau, the 2nd insert appears to decrease the ability of Tau to pass the TDB. Considering that 2N3R-Tau is bigger than 0N4R-Tau, also increased size would correlate with inability to cross the TDB. This idea would be in agreement with a previously proposed unspecific size-dependent filter (26,27). However, mutants of the longest isoform of Tau, derived from FTLD or PSP (i.e. ΔK280 and A152T), and also a pseudophosphorylation mutant (4KxGE, all 4 KxGS motifs mutated to KxGE) show enhanced retrograde and anterograde passage of Tau, indicating that size alone is not the sole determinant.

b) Tau increases spine development and dendritic growth in an isoform dependent manner

The longest isoform of Tau (2N4R-Tau) along with the other 4R isoforms is expressed only after the initial polarization has taken place, i.e. not during embryonal development, both in rodents and humans. When expressed in low endogenous-like levels, some Tau isoforms (e.g. 3R Tau species, especially 0N3R) induce filopodia-like spine growth. Other isoforms (0N4R, 1N4R) had no or little effect on promoting spine development. 2N4R-Tau, however, induces striking maturation of spines and elongation of dendrites (Fig.2D-F). This is in line with the appearance of specific Tau isoforms during development: The short (3R) isoforms are expressed early in development driving filopodia spine formation while later in life the longer isoforms (e.g. 2N4R) are expressed (6), which concurs with spine maturation. Accordingly, knockdown of Tau at the time of spine formation and maturation (14DIV), results in loss of spine formation. This points towards a bona fide role for Tau in dendritic development. Future studies, e.g. by knocking down individual isoforms of Tau while maintaining the physiologogical ratio of the remaining isoforms will have to elucidate which isoform of Tau during which state of development is responsible or contributes to normal spine development.

c) Sorting of Tau can be achieved independently of regulatory translation, but is modulated by phosphorylation of Tau and AIS structural integrity

We studied the mechanistic/structural manner how the TDB is able to confine the Tau protein to the axonal compartment. Previously, Tau mRNA was shown to be translocated to the axon by an 3'UTR axonal targeting signal, implying local translation of Tau (20). However, the exogenous Tau constructs we used to investigate the TDB and the axonal enrichment do not carry the 3'UTR. Thus, in our model, Tau mRNA does not contribute to the sorting of Tau.

Phosphorylation of Tau has likely a function in sorting of Tau: Pseudophosphorylation at the KxGS motifs enhances anterograde and retrograde propagation of Tau in the axon (22,54). Activation of the kinase MARK (primarily responsible for phosphorylation of these epitopes) lags behind Aβ-induced missorting of Tau, indicative of a role in resorting of Tau. Here we show that Tau pseudophosphorylated at the KxGS motifs shows enhanced axonal enrichment compared to wildtype Tau (Fig.2,S1,2), indicating the importance of this phospho-site for sorting. Another prominent Tau kinase, GSK3β, is a major player within the AIS where it is anchored (35), but does not phosphorylate KxGS motifs of Tau. Testing the influence of GSK3β on the TDB revealed that GSKβ induces accumulation of endogenous and exogenous Tau in the somatodendritic compartment (Fig.3,S5). In case of retrograde missorting of exogenous Tau this effect is independent of direct phosphorylation of Tau by GSK3β, because even non-phosphorylatable Tau shows the same retrograde propagation from the axon into the soma (Fig.3). Thus, the interplay of GSK3β with components of the AIS/TDB is essential for sorting and missorting of Tau in our model. Knockdown of other structural components of the AIS results in weakening of the TDB. Tau transit through the TDB is facilitated when classical AIS components (e.g. AnkG, EB1) are knocked down via shRNA. All knockdowns also induce increased invasion of the dendritic marker MAP2 into the proximal part of the axon. This indicates that the classical AIS components contribute to the TDB, but also to the nonspecific filter function of the AIS.

d) Structures around the TDB: Highly dynamic MTs in the AIS

MT destabilization by nocodazol results in missorting and breakdown of the TDB, whereas MT stabilization by taxol prevents missorting after Aβ-insult (22,49). We thus hypothesized that the structure of MTs within the AIS holds the key to the TDB. Surprisingly, MTs within the AIS are extremely dynamic. Markers of stable MTs (acetylation, polyglutamylation) are absent in the AIS. MTs could not be stained in the AIS without the use of the molecular densifier polyethylene glycol (PEG) and taxol, emphasizing the transient nature of these MTs (Fig.4), similarily to previous studies (31). EB3-based live-imaging of MT dynamics confirmed the dynamic nature of AIS MTs and revealed a hub of EB3-comet spawning at the central part of the AIS (Fig.5), consistent with observations of enrichment of EB-proteins in the AIS (28).

Of note, EB3-comet speed (and thus MT growth rates) is unchanged in the different parts of the AIS and also in the axon (constant at ~0.5μm/s for the proximal axon, the AIS itself and the axon hillock), only after an AD-like insult with Aβ oligomers the EB3-comet speed decreases by ~20% (Fig.7B).

e) AD-like insult by Aβ causes axodendritic missorting of Tau due to activation of cofilin and loss of MT-growth within the AIS

Aβ oligomers bind preferentially to dendritic spines. However, within axons the AIS is a preferential target for Aβ (Fig.S8). As the AIS is composed of a multitude of regulatory elements and receptors (55), Aβ binding may impair the function of the AIS. This is indeed the case for the TDB, which lies within the AIS. Aβ exposure causes activation of cofilin, resulting in increased severing, loss and subsequent remodeling of F-actin. While this leads to increased amounts of F-actin in

the dendritic shaft likely due to redistribution from shriveling spines, the first effect in the AIS is a decrease of F-actin.Thus the observed activation of cofilin and consequent loss of F-actin particularly within the AIS perturbs the F-actin meshwork in the AIS. This meshwork acts as a filter to retain non-axonal proteins in the soma. The structural organization appears different depending on the techniques employed, but likely consists of spectrin IV bound regularly spaced F-actin rings, still, a large portion of the actin cytoskeleton in the AIS is thought to be dynamic (56,57). In case of the TDB, breakdown of F-actin results in anterograde penetration of the "dendritic" 2N4R-Tau, but also failure of proper sorting into the axon of the stronger axonally sorted 0N4R-Tau (Fig.6,7). Thus, the failure of the TDB results in axodendritic missorting of Tau that is isoform specific.

Aβ-induced loss of F-actin within the AIS may also result in facilitated diffusion of other proteins: Dendra2 e.g. shows ~2x faster diffusion into the axon. Aβ-induced disruption of F-actin within the AIS would explain the increased anterograde propagation of the longest isoform of Tau, but why is the normally strongly axonally targeted major isoform of Tau (0N4R) missorted after Aβ insult (Fig.7)? First, since Aβ disrupts also the anterograde polymerization of MTs within the AIS, this would decrease the pumping mechanism which transports the Tau-tubulin complex into the axon (54). Second, as the peak of missorting of Tau (3-6h, (49)) lags substantially behind the loss of F-actin and activation of cofilin (0.1-1h, Fig.6), likely the subsequent (aberrant) remodeling of F-actin is responsible for missorting, rather than the loss of F-actin per se. Consistently, the F-actin stabilizer Jasplikanolide induces missorting, but not the F-actin destabilizer Latrunculin.

Remodeling of the F-actin cytoskeleton within the AIS would result also in the impairment of MT polymerization within the center of the AIS, although it is currently unclear whether there is a direct causal link and whether there is an effector protein. Anyhow, the remodeling of F-actin within the AIS does not occur prior to 1h after Aβ exposure. Thus, strangulation of MT growth by aberrant F-actin repolymerization within the AIS would start after Aβ has lost its toxicity, and F-actin starts to repolymerize. This we indeed observed (Fig.6,7A). Tau is then unable to traffic along MTs, decreasing its ability to pass the anterograde TDB, resulting in somatodendritic accumulation (classical missorting). The cause-effect relationship however is far from obvious, as it was shown recently that Tau stabilizes MT growth, judged by EB1- and EB3-comet number and comet path length (58). Thus the reduced entry of Tau into the AIS (due to aberrant F-actin remodeling) may also contribute to reduced MT growth.

f) Conclusion

We show here that the barrier function of the Tau diffusion barrier (TDB) within the AIS can operate both in the anterograde and retrograde direction. For 90-97% of Tau molecules where the second insert is absent, Tau passes through the TDB anterogradely and is retained in the axon. In case of the longest isoform 2N4R (~3-10% of Tau), Tau molecules are partially retained in the somatodendritic compartment where they enhance dendritic outgrowth and spine formation. Passage of the TDB barrier in both directions is facilitated when Tau itself is mutated, i.e. in case of pathological mutations, phosphorylation, knockdown of AIS components or overexpression of GSK3β (an AIS and AD-kinase). Pathomechanistically, we show that AD-like Aβ insult leads to cofilin activation and remodeling of F-actin within the AIS, resulting in decreased MT mobility. This leads to somatodendritic accumulation of 0N4R-Tau, hence classical missorting, but also enhanced axonal penetration of 2N4R-Tau. In summary, we provide evidence for physiological and pathomechanistical differences of the different Tau isoforms, their role in sorting and missorting, and demonstrate functional importance for MT and actin dynamics within the AIS for the maintenance of the TDB.

Materials and Methods

Cell Culture and Transfection: Dissociated cortical neurons were prepared from embryonic day 18 (E18) Wistar rats or E16 C57BL6JRj wildtype or TauKO mice according (59) and plated on poly-D-lysine (50 µg/ml)-coated cover glass. After 4 days *in vitro* (DIV), 0.3 µM cytosine arabinoside (Sigma, Munich, Germany) was added to the cultures to reduce glial growth. Cultures were maintained in NeuroBasal medium with B27 (Invitrogen) or NS21 (PanBiotech), L-glutamine (PAA) and Penicillin/Streptomycin (PAA/ PanBiotech) at 37°C, 5% CO2. Lipofectamine based transfection for experiments for retrograde Tau movement was conducted as described previously in (22) (high protein expression), for experiments investigating the Tau distribution after days of low expression was conductes as described in (60) making use of standard Lipofectamine 2000 (L2K; Invitrogen) reagent, but not OptiMEM. Briefly, for low protein expression, DNA amount was limited to 1,2µg/construct/well of a 6-well multiwell plate or to 0.6µg/construct/well of 12 or 24 well plates. Instead of OptiMEM, pure unsupplemented Neurobasal medium (Gibco) was used. Other than that L2K/DNA mix was prepared as recommended (5min+30min incubation). Next, cell culture medium already in the multi well plates (condition medium) was carefully reduced to 1ml for 6 well plates, or 0.5ml for 12 and 24 well plates, saving the remaining conditioned medium. Cells were then incubated for 6h for cells older than 14DIV or 3h for cells between 7 and 10DIV) with the L2K transfection mix. Next, cell were washed twice in the multiwell plates with preheated and carboxynated unsupplemented neurobasal medium, taking great care that cells never fell dry (critically important for older cells). Next the earlier removed conditioned medium was used to allow expression of transfected constructs for 4-6 days. Exposure of cells to reagents and media other than conditioned medium was kept to the absolute minimum to avoid cell degeneration (see also (60) for critical steps and modulation of expression).

Plasmid construction: The cloning of Dendra2-htau40 was reported previously (22). Cloning of various isoforms of Tau was done either by PCR-based amplification or subcloning of restriction digested fragments into the respective restriction sites. PS-CFP2 vector was purchased from Evrogen and cDNA of human full-length Tau was subcloned into the C-terminal of PS-CFP2. Tagging of Tau was tested with several tags (PS-CFP2, Dendra2, CFP, HA) and without tags, without noticeable differences in cellular distribution (FigS9). Cloning of various shRNAs was performed in pShuttleCMV/mRFP/siRNA/H1RNAPromotor plasmid. The sequence of shRNAs used is as follows: Nav1.x, 5′-GTTCGACCCTGACGCCACT-3′; ankG, 5′-GCCGTCAGTACCATCTTCT-3′; βIV spectrin, 5′-CACTGGATAGCCGAGAAGG-3′; NrCAM, 5’-CAATAATCCTCCGAAGTG-3’; EB1 5’-TCTGACAAAGATAGAACAG-3’; EB3 5’-ACTATGATGGAAAGGATTAC-3’and spastin 5’-GGAAUGGUAUAAGAAAGGUAUCGAA-3’;. All plasmids were verified by restriction analysis and DNA sequencing. Successful knockdown is demonstrated in (61-64). Adenovirus production and transfection was as described (65).

Imaging, photoconversion, and analysis: A Fluoview1000 confocal microscope (Olympus, Hamburg, Germany) equipped with a SIM scanner (which allows for simultaneous stimulation and imaging) and a × 60 objective live-cell imaging chamber and ZDC system for Z-drift compensation was used for photoconversion experiments and image acquisition. For time-lapse live-cell imaging, cells were plated on glass bottom dishes and were kept in the imaging chamber (37°C, 65% humidity and supplied with 5% CO2). Images were taken every 30 s for all applications. Before photoconversion, green fluorescence of Dendra2 was acquired by excitation at 488nm and detection over the range of 500-550nm. Photoconversion was performed during the imaging interval by rapidly illuminating the selected area with a 405-nm laser scanner (1–2 exposures of 1s each, 405nm laser power 15%). Thereafter, red fluorescence was acquired by excitation at

561nm and detection over the range 570-670nm. In case of PS-CFP2 protein, cyan fluorescence was acquired by excitation at 405nm with 0.2% laser power and detection over the range of 420-510nm. Photoconversion was performed similar to Dendra2, and green fluorescence of PS-CFP2 was acquired by excitation at 488nm and detection over the range of 500-550nm. For comparison of Dendra2 and PSCFP, fluorescence intensities were normalized using Dendra2 or PS-CFP2 alone and hTau40 tagged with the different fluorescent proteins as controls; fluorescent photoswitchable proteins (PSCFP2 and Dendra2) cell body intensities after axonal conversion were set to 100, and baseline levels were normalized by noise subtraction. For knockdown of the different AIS components PSCFP2 was used because knockdown vectors contained mRFP, which had to be used to identify cells expressing the shRNA. Fluorescence intensity profiles and values according to the indicated time points and area were determined by the microscopy software (Olympus FV1000 Software). Microscopic settings as well as the selection of the regions of interest were kept similar in order to allow comparison between different experiments as described previously (22). STED imaging was conducted as recommended by the manufacturer (Leica) with a gSTED system on a TCS SP8 secondary antibodies conjugated with CF488 (VWR) or Alexa488 (Dianova) as chromophores, and AntifadeGold mounting medium (Invitrogen). Images were analysed using manufacturer software (Leica LAS AF Lite). Standard immunofluorescence was conducted essentially as described before (12,49). Axons were identified by absence of MAP2 and presence of Tau staining, branching pattern of ~90°, recurrence to cell body and crossing of processes of the same cell, length of above 300µm and constant diameter. Dendrites were identified by positive MAP2 stain, presence of dendritic spines, branching pattern of ~60°, constantly decreasing diameter, and a length of below 300µm. Cell bodies, dendrites and axons have very different diameters, which is impossible to judge by light microscopy. We therefore used a volume marker (tdTomato) and compared intensities of transfected constructs (i.e. Tau^{D2} stained with human Tau specific antibody CP27) to the intensities of the volume marker. The volume marker was assumed to be distributed throughout the cell in an unbiased manner, thus making comparisons of the different Tau constructs possible. Values given in results section and figures were calculated by dividing the normalized (i.e. CP27 fluorescence intensities (Tau) vs. tdTomato (Tom) fluorescence intensities) axonal values (AV) vs. dendritic values (DV). The formula therefor is $(Tau^{AV}/Tom^{AV})/(Tau^{DV})/(Tom^{DV})$; for illustration purposes values were multiplied by factor of 50 (to attain a maximal value of 100).

For the evaluation of spine development after transfection of different Tau isoforms and mutations leading to Figure 2D and Table S1 at least 3 cultures from each genotype of wildtype and TauKO hippocampal neurons and several cultures from rat cortical and hippocampal neurons were included.

MT stabilization during extraction was conducted using 3min extraction at room temperature, in MT stabilizing buffer ((PEM buffer: 2% polyethylene glycol (MW:10.000) 100mM PIPES-KOH, ph 6.9, 1mM MgCl2, 1mM EGTA) containing 1% Triton-X-100 and 2µM taxol (all Sigma or VWR), after a quick rinse in PBS (PAA/Sigma). Cells were then fixed with 3.7% formaldehyde and 0.5% glutaraldehyde in PEM-buffer for 20min. Mitochondria and EB3 live-imaging was conducted at 2s intervals with a MM-based Leica DM6000, processed and analysed with Metamorph software.

Other procedures: Western blotting, ATP and LDH measurements, and statistical analyisis (* indicates $p<0.05$, ** indicates $p<0.01$ vs control if not indicated otherwise) were done as described before (12,49). Dephosphorylation of endogenous rat Tau without phosphatase inhibitors but with protease inhibitors, making use of alkaline phosphate (FastAP;ThermoFisher Scientific) for 16h. Oligomeric Amyloid-beta was prepared as described before in (49). Briefly, Abeta 40 and Abeta 42 (both 21^{st} peptide) were mixed in a 7:3 ratio and oligomerized via

sequential dissolution in 40mM NaOH to a 2mM Abeta solution, and consequently diluted in PBS to a 100µM solution. This solution was then incubated for 1h at 37°C. For complete characterization and differences to unmixed preparations see (49).

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

YK, FD, EMM, EM and HZ conceived the study, HZ, EM, FD and EMM wrote the paper. YK conducted the experiments leading to Fig.1 and Fig.S3A,B, data was processed and illustrated by FD and HZ. FD and HZ jointly designed and conducted experiments leading to Fig.3 and Fig.7D. JB designed genetic constructs and provided critical help with molecular biology. All other experiments were conceived, conducted, processed and illustrated by JL and HZ.

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Tables

Table 1: mRNA and protein levels of the different isoforms of Tau in human and mouse brain

Tau clone names (3)	analogous Tau isoform N: inserts, R: repeats	isoform fraction adult **human mRNA** (4)	isoform fraction adult **human protein (cortex)*** (5)	isoform fraction adult **mouse mRNA** (6)	isoform fraction adult **mouse protein** (6)
hTau40	2N4R	3	8 (+/-7)	38	38
hTau34	1N4R	24	23	30	41
hTau24	0N4R	23	13	32	21
hTau39	2N3R	4	3	n.a.	n.a.
hTau37	1N3R	25	26	n.a.	n.a.
hTau23	0N3R	21	19	<1	<1

*Note that values in column 4 do not add up to 100%, likely due to sample variation in (Trabzuni et al., 2012).

Figure Legends

Figure 1: The 2nd repeat and inserts synergistically prevent Tau from retrograde passage through the TDB in the AIS while mutations cause retrograde leakage into the soma.
(A) Bar diagrams of the studied isoforms of Tau. Alternatively spliced N-terminal inserts (N1, N2) in red, repeat domain green, repeat R2 yellow. Mutated 2N4R-Tau species are displayed in the lower half, mutations represented by red vertical lines.
(B) Photoconversion of primary rat cortical neurons transfected with control 2N4R-Tau^{D2} (top) or 2N4R-Tau^{D2}-KXGE mutant (bottom). Panel 1&5: Reference image of unconverted protein before photoconversion to identify soma and axon in the green channel. Panel 2&6: Before photoconversion there is no fluorescence in the red channel. Panels 3&4a: After photoconversion in region of interest (dotted box, red), cells transfected with 2N4R-Tau^{D2} (top) show movement of Tau^{D2} beyond the ROI but not into the soma (red triangle) and dendrites, indicating a retrograde barrier in the initial part of the axon. Panel 7&8a: In contrast, cells transfected with 2N4R-Tau^{D2}-KxGE mutant show penetration of photoconverted protein into the somatodendritic compartment. Pictograms (4b&8b): graphical representation of 2N4R-Tau^{D2} unable to cross the Diffusion barrier within the AIS (4b, cell body remains dark) and 4KxGE-Tau^{D2} which crosses the AIS and reaches the cell soma (8b, cell body lights up).
(C) Level of Tau leaking through the barrier into the soma after photoconversion shown for several Tau isoforms (blue) and Tau mutations (red). Isoforms lacking both the second repeat (R2) and the second insert (N2) penetrate into the somatodendritic compartment. Pseudophosphorylated Tau (KxGE) or FTLD-Tau related mutations (A152T and ΔK280) also cause incomplete axonal retention. Note that the tightness of the barrier does not depend on the propensity of Tau for β-structure that controls aggregation (compare ΔK280, ΔK280PP).
(D) Decay of a selection of photoconverted Dendra2 and Tau^{D2} species in the photoconverted area of the axon. Axonal diffusion rates correlate with the MT binding potential of the species involved in some cases (e.g. Dendra2→low and 8 repeat Tau→high), but not in case of FTLD-related Tau mutations. Note that the half live of 2N4R-Tau is roughly intermediate between 8-repeat-Tau and KxGE-Tau (indicated by dotted colored lines).
(E) Quantification of the half lives in the photoconverted area of the axon (distal to the AIS) of all Tau isoforms including 2N4R Tau (§), Dendra2 alone (†), 8 repeat Tau(‡) as well as KXGE Tau (•), A152T Tau, Tau ΔK280 and Tau ΔK280PP.
Bars marked with Dendra2 alone (†), 8 repeat Tau (‡), 2N4R Tau (§) and KXGE Tau (•) are the quantifications of the decay curves displayed in figure 1D.
*indicates $P<0.05$

Figure 2: 2N4R-Tau is retained in the somatodendritic compartment in wildtype neurons, where it induces accelerated spine and dendrite growth, whereas 0N4R-Tau can enter axons

Different isoforms of Tau^{D2} were cotransfected with tdTomato (volume marker) for 6 days into primary neurons (7DIV) derived from TauKO or wildtype mice.

(A, B) Tau^{D2} transfected into TauKO cells (A) and wildtype cells (B). Transfected Tau was stained with an antibody against human Tau (CP27). Arrowheads indicate the axon, arrows indicate dendrites.

(A) 0N4R-Tau shows strong enrichment in axons (for more constructs see Fig.S1).

(B) Wildtype neurons expressing endogenous Tau in addition to transfected exogenous Tau^{D2} (stained with CP27). Exogenous and endogenous Tau is stained with a panTau antibody (K9JA), dendrites are stained with an antibody against MAP2.

Columns 1, 2: 0N4R-Tau is enriched in the axon. As a result, axonal Tau concentration is higher than in neighboring untransfected axons where only endogenous Tau is present, but can barely be detected in dendrites with a panTau antibody. Col 2, bottom shows magnified images from boxed areas, axon is indicated by arrowheads.

Columns 3, 4: 2N4R-Tau is not enriched in axons.

Column 5: 2N4R-Tau pseudophosphorylated at the KxGS-motifs (KxGS-motifs mutated to KxGE) shows strong axonal enrichment.

(C1) Quantification of axonal enrichment of different Tau^{D2} isoforms and 2N4R-Tau^{D2} mutations reveal lower axonal presence of the longest isoform of Tau (2N4R) compared to the other isoforms. The exception is the human-specific isoform 1N3R, not present in rodents. The mutations 4KxGE, A152T, and ΔK280PP of 2N4R-Tau^{D2} increase the axonal sorting, while the mutation ΔK280 has no effect.

(C2) Graphical sketch of the different distributions of the representative isoforms 0N4R (strong axonal enrichment) and 2N4R (weak axonal enrichment). Left panels: Tau distribution depicted in green. Right panels: Tau distribution in green presented relative to a volume marker with unbiased distribution in red, resulting in a ratiometric image.

(D,E) Wildtype neurons with tdTomato as an unbiased volume/morphology marker.

(D) Tau^{D2} transfection induces spine formation and maturation in young (10 DIV; panels 1-6) neurons, Tau knockdown abolishes spine maturation in old (21 DIV; panels 7,8) neurons.

(1) Neurons only transfected with a control vector (Dendra2) show no spine formation and very few filopodia, representative of neurons on the brink of - but before - spine development.

(2) Transfection of 0N3R-Tau results in elongated filopodia-like protrusions up to 10µm;

(3) by contrast 0N4R-Tau has no effect on dendritic spine/filopodia formation.

(4) 2N4R-Tau transfected cells show dendritic processes with mushroom heads (arrowheads), indicative of mature spines.

(5, 6) Mutations of the KxGS motifs in the repeat domain to KxGE (5) or KxGA (6) of 2N4R-Tau prevents formation of protrusions and acceleration of spine maturation. For a comprehensive list of effects of all isoforms and several mutations see table S1.

(7) Aged control neurons display normal mature spines.

(8) Aged neurons transfected with shRNA against Tau display impaired spines.

(E1) Tau transfection induces dendritic outgrowth.

Left panel: Neuron transfected with a control vector (Dendra2). Right panel: Neuron transfected with the 2N4R-Tau. Longest dendrites are indicated by red dashes.

(E2) Quantification of the longest dendrite of cells transfected as indicated.

(F) Graphical sketch of the dendritic effects of the representative Tau isoforms 0N3R (filopodia spines), 0N4R (no dendritic effects) and 2N4R (elongated dendrites and premature development of spines).

*indicates $P<0.05$

Figure 3: GSK3β induces missorting of Tau independently of phosphorylation of Tau's SP/TP motifs.
Primary cortical rat neurons (7DIV) were cotransfected for 3d with different versions of 2N4R-Tau^{D2} and GSK3β (tagged with CFP). Panels A1,5,9 show cells in the green channel before photoconversion (PC), panels A2,6,10 show cells in the red channel before PC, and panels A3,4,7,8,11,12 after PC at indicated times. Scale bars: 20µm.
(A1-4) Primary rat cortical neurons (7DIV) cotransfected with Tau^{D2} and GSK3β(wt) show leakage of the Tau diffusion barrier and retrograde propagation of Tau back into the cell body (quantified in B black bar).
(A5-8) Inactive GSK3β-S9E does not cause leakage since Tau^{D2} is retained in the axon and does not move into the cell body (quantified in B, red bar).
(A9-12) Mutating the GSK3β target sites of Tau to alanine (17 SP/TP motifs mutated to AP, AP17-Tau^{D2}) results in a leaky barrier so that AP17-Tau^{D2} still shows retrograde propagation back into the cell body (quantified in B, purple bar).
(B) Quantification of A as indicated.
(C) Being unable to be phosphorylated at its SP/TP motifs, AP17-Tau reveals to have a very slow diffusion rate, purple trace. Penetration of the TDB can be seen nonetheless as was shown in panel A9-12 and B purple bar.

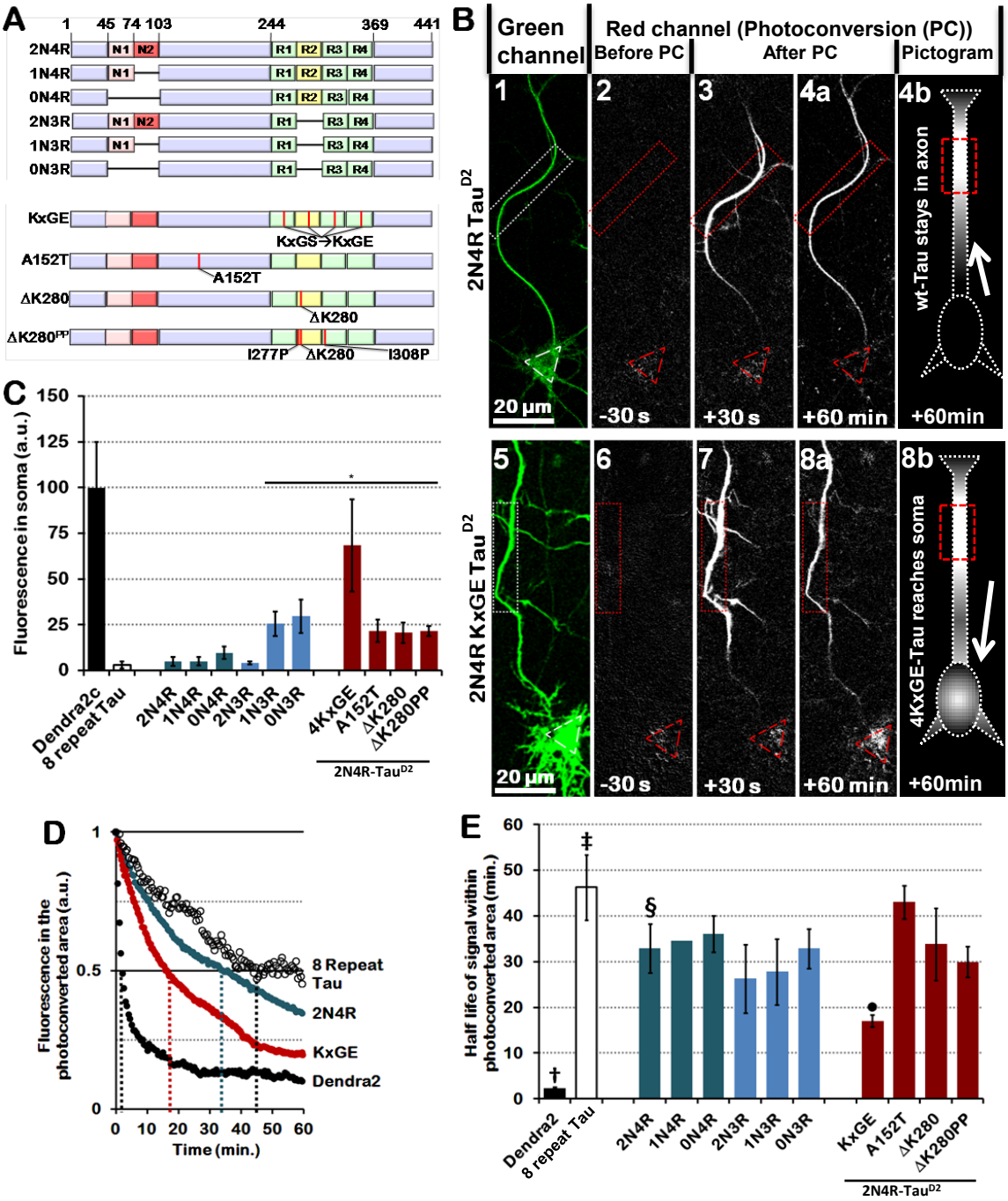
Figure 4: Posttranslational modifications of MTs and STED-nanoscopy demonstrate highly dynamic MTs within the AIS.
Primary cortical neurons were fixed at 9-12DIV and stained as indicated. The AIS is marked by dotted lines.
(A) Examples of images of cells highlighting axon initial segments. Axons are marked by arrowheads, dendrites by arrows. Asterisks mark staining artefacts.
(A1,2) Double staining with MAP2 (blue) as a dendritic marker and NrCam (red) as an AIS marker allows identification of the AIS. Acetylated tubulin (green) was used as an axonal marker, highlighting stable MTs. Inserts show graphical sketches. **(A3)** Magnification of boxed area of A2. Note that there is no signal for acetylated tubulin within the AIS.
(B-D) Quantification of immunofluorescence intensities from the middle of the cell body over the AIS to the proximal axon. Red vertical line indicates beginning of the AIS. Right section of graphs indicate distal axons. On average, the AIS begins about 15-20 µm from the center of the cell body and extends for about 20-30 µm as the AIS markers gradually change towards their values in the proximal axon.
(B) The beginning of the AIS is characterized by a decrease of MAP2 (blue) and an increase of NrCAM (red).
(C) Levels of tyrosinated tubulin (corresponding to dynamic MTs, red) are lower in the AIS than in the cell body, but higher than in the distal axons. Levels of acetylated tubulin are low at the proximal AIS.
(D) Different fixation and extraction procedures were used for preferred observation of unpolymerized tubulin (green) or MTs (brown) (see Methods). Levels of free tubulin are high within the AIS, yet levels of MTs are low, indicating high levels of unpolymerized tubulin.
(E,F,G) STED nanoscopy of MTs within the AIS.
(E, F) Normal fixation/extraction method to favor stable MTs. **(E1, F1)** There are no MTs stainable with an antibody against α-tubulin (E1) and very few fragmented MTs with an antibody against tyrosinated (dynamic) MTs (F1) within the AIS (space between dotted lines). **(E2,F2)** NrCam was used for identification of the AIS.
(G) Extraction and fixation were conducted in buffers containing the MT stabilizer taxol and the molecular densifier PEG. Left panels: NrCam and MAP2 were used to identify the AIS. Arrow indicates point of increasing NrCam signal and decreasing MAP2 signal, hence the beginning of the AIS. Right panel: High density of MTs within the AIS stained by an antibody against tyrosinated (dynamic) MTs, arrow at the same position as left panels.

Figure 5: EB3 clusters in the proximal region of the AIS
Primary cortical neurons aged 9DIV were transfected with EB3-GFP for 2d
(A,B) Live-imaging of EB3 and time-resolved kymographs of the AIS.
(A) EB3-comets are enriched and dynamic within the AIS. Upper panel: First frame of time lapse imaging of the AIS and the proximal axon. Arrow indicates thickening of the AIS typically present over 5-10µm within the AIS. Lower panel: Kymograph of the EB3 comets show unidirectional movement only anterogradely, typical of the axon, and comets within the AIS-thickening.
(B) Analogous region as in (A), but in a proximal dendrite. Note that EB3 comets move in both directions, and there is no thickening or area of enrichment of EB3.
(C) Cells were fixed 2d after transfection with mitoRFP and stained for MAP2 and EB3 for identification of the AIS. Arrow indicates beginning of AIS (end of MAP2 staining) and mitochondrial agglomeration proximal to the AIS, arrowhead indicates thickening of the AIS and enrichment with EB3 distal to the mitochondrial cluster.
(D) Graphical sketch of mitochondrial and EB3 positioning at the AIS. Mitochondria cluster at the proximal end of the AIS (where MAP2 diminishes), while EB3 staining is localized within the AIS.

Figure 6: Aβ exposure results in cofilin activation and loss of F-actin within the AIS
Primary neurons 21DIV were treated with 1µM Aβ as indicated, then fixed and stained as indicated
(A) Co-staining of the AIS with phospho-cofilin (via a phosphorylation-dependent antibody) and filamentous actin (F-actin, stained via phalloidin) shows positive stainings in control conditions (A1), but reveals a loss p-cofilin (dephosphorylation results in activation of cofilin and severing of F-actin) and loss of F-actin signal after 15min of Aβ exposure (A2).
(B) Quantification of p-cofilin signals (B1) and F-actin signals (B2).

Figure 7: Aβ disrupts the Tau Diffusion barrier via impairment of MT-dynamics in the AIS
(A) Kymograph (A1) of the AIS of a f-tractin^{Citrine} transfected primary cortical neuron (12DIV) for 4d after treatment with Aβ. The F-actin labeling protein f-tractin^{Citrine} accumulates within the AIS, indicating an increase in area covered by F-actin. Boxed areas indicate the regions of higher F-actin concentrations within the AIS. Arrows indicate increasing F-actin (1-1.5h). Quantification (B2) reveals a constant increase in fluorescence intensity (FI) of f-tractine^{Citrine} after 0.5h exposure to Aβ.
(B) Kymographs (B1) of the AIS of EB3-tdTomate transfected primary cortical neurons 11DIV untreated (upper panel) or treated with Aβ (1µM) for 1.5h. Quantification (B2) reveals decreased speed of EB3 comets only within the AIS.
(C) Treatment of primary neurons (20DIV) with Jasplakinolide (Jaspl.) and Aβ, but not Latrunculin-A (LatA), results in missorting of endogenous Tau. Neurons were treated for 3h as indicated and stained with K9JA for total Tau (green color). **(C1)** Control neurons show little Tau presence in the somatodendritic compartment (left panel), while treatment with Jasplakinolide (1µM) results in increased Tau presence in the somatodendritic compartment. cb: cell body, d: dendrite.
(C2) Quantification of missorting of Tau as indicated.
(D) 2N4R-Tau^{D2} and 0N4R-Tau^{D2} as well as Dendra2 were transfected into primary rat cortical neurons (8 DIV) for 3d. **(D1)** Images depict neurons 10min post-photoconversion in the soma, inserts show cells before photoconversion and distribution of the unconverted protein in green. Left panel: 0N4R-Tau^{D2} transfected neurons show fast anterior movement of photoconverted 0N4R-TauD2. Right panel: Treatment with 1µM Aβ for 1h reduces axonal sorting of 0N4R-Tau^{D2}.
(D2) Quantification of experiments as shown in (D1). Cells transfected with Dendra2 alone or 0N4R-Tau^{D2} show anterograde penetration of the TDB while 2N4R-Tau^{D2} shows reduced anterograde movement into the axon in control conditions (black bars). Treatment with Aβ for 1h reduces retention of Dendra2 and 2N4R-Tau^{D2}, but shows a trend for enhanced retention of 0N4R-Tau^{D2}.
(E) Axonal sorting of the 0N4R-Tau is disrupted by Aβ treatment. Primary cortical neurons 16DIV were transfected with the different rodent isoforms of Tau tagged with Citrine (mTau^{Cit}, green color) and the volume marker tdTomato (red color) for 5d. **(E1)** 2N4R- mTau^{Cit} shows little enrichement in the axon (left panels) while the 0N4R-mTau^{Cit} isoform is enriched in the axon (middle panels, arrow, green color), but this enrichment is lost after Aβ treatment (right panels).
(E2) Quantification of the axonal enrichment of all rodent Tau isoforms tagged to citrine (mTau^{Cit}) with and without treatment with 1µM Aβ for 3h. The shorter 0N isoforms are enriched in the axon (arrow), but this enrichment is partially lost after Aβ treatment.
*indicates P<0.05; **indicates P<0.01

Figure 1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2N4R
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N2
R1
R2
R3
R4
KxGE
KxGS→KxGE
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ΔK280PP
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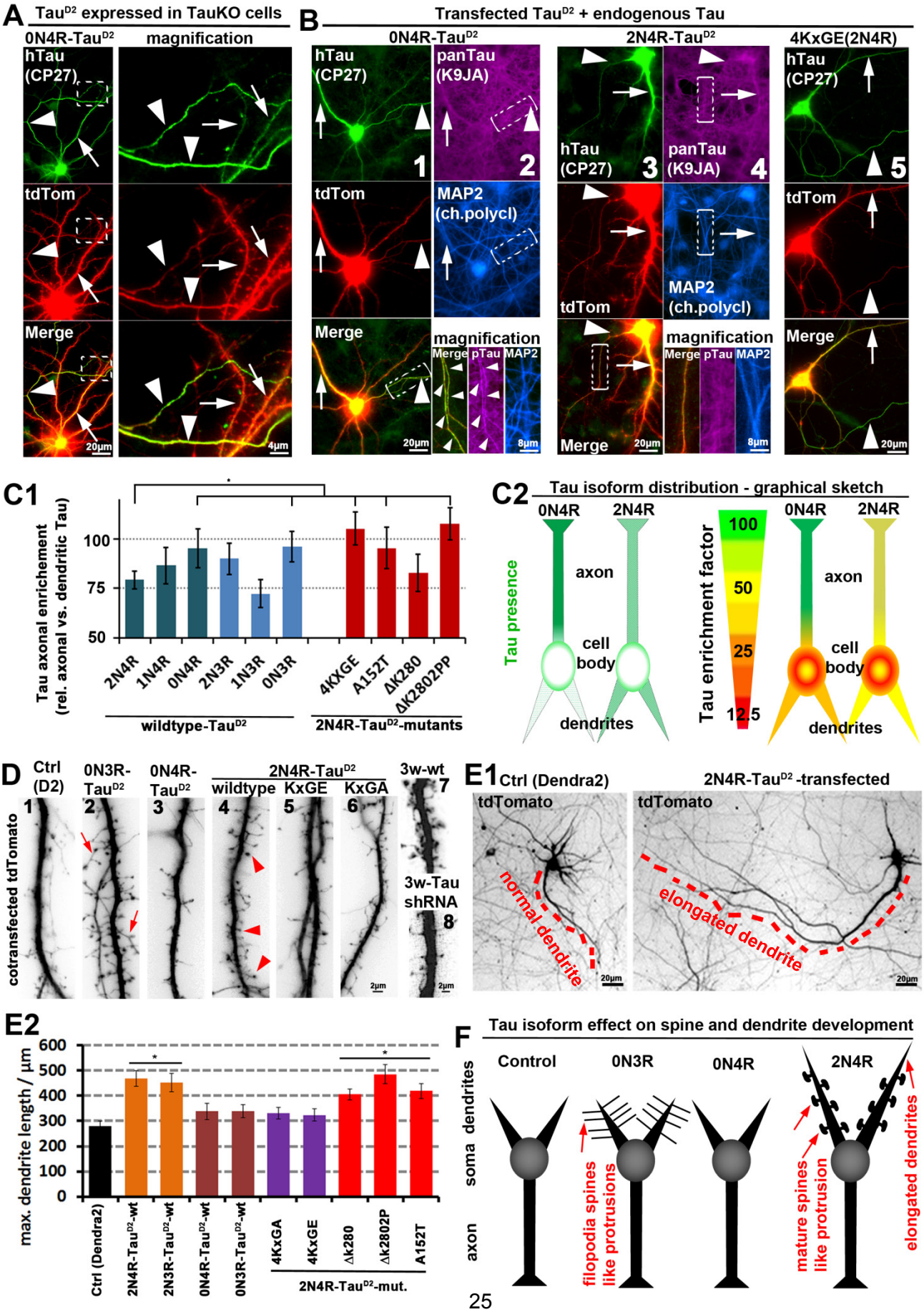
I308P
B
Green channel
Red channel (Photoconversion (PC))
Before PC
After PC
Pictogram
2N4R Tau D2
2N4R KxGE Tau D2
20 µm
-30 s
+30 s
+60 min
+60min
wt-Tau stays in axon
4KxGE-Tau reaches soma
C
Fluorescence in soma (a.u.)
Dendra2c 8 repeat Tau
4KxGE
2N4R-Tau D2
D
Fluorescence in the photoconverted area (a.u.)
Time (min.)
8 Repeat Tau
2N4R
KxGE
Dendra2
E
Half life of signal within photoconverted area (min.)
Dendra2
8 repeat Tau
KxGE
2N4R-Tau D2

Figure 2


A
Tau^D2 expressed in TauKO cells
0N4R-Tau^D2
magnification
hTau (CP27)
tdTom
Merge
20µm
4µm
B
Transfected Tau^D2 + endogenous Tau
0N4R-Tau^D2
2N4R-Tau^D2
4KxGE(2N4R)
hTau (CP27)
panTau (K9JA)
MAP2 (ch.polycl)
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3
4
5
20µm
8µm
C1
Tau axonal enrichement (rel. axonal vs. dendritic Tau)
100
75
50
2N4R
1N4R
0N4R
2N3R
1N3R
0N3R
4KXGE
A152T
ΔK280
ΔK280PP
wildtype-Tau^D2
2N4R-Tau^D2-mutants
C2
Tau isoform distribution - graphical sketch
0N4R
2N4R
Tau presence
axon
cell body
dendrites
Tau enrichment factor
100
50
25
12.5
D
Ctrl (D2)
0N3R-Tau^D2
0N4R-Tau^D2
2N4R-Tau^D2
wildtype
KxGE
KxGA
3w-wt
3w-Tau shRNA
cotransfected tdTomato
1
2
3
4
5
6
7
8
2µm
E1
Ctrl (Dendra2)
2N4R-Tau^D2 -transfected
tdTomato
normal dendrite
elongated dendrite
20µm
E2
max. dendrite length / µm
600
500
400
300
200
100
0
Ctrl (Dendra2)
2N4R-Tau^D2-wt
2N3R-Tau^D2-wt
0N4R-Tau^D2-wt
0N3R-Tau^D2-wt
4KxGA
4KxGE
Δk280
Δk2802P
A152T
2N4R-Tau^D2-mut.
F
Tau isoform effect on spine and dendrite development
Control
0N3R
0N4R
2N4R
soma dendrites
axon
filopodia spines like protrusions
mature spines like protrusion
elongated dendrites

Figure 3

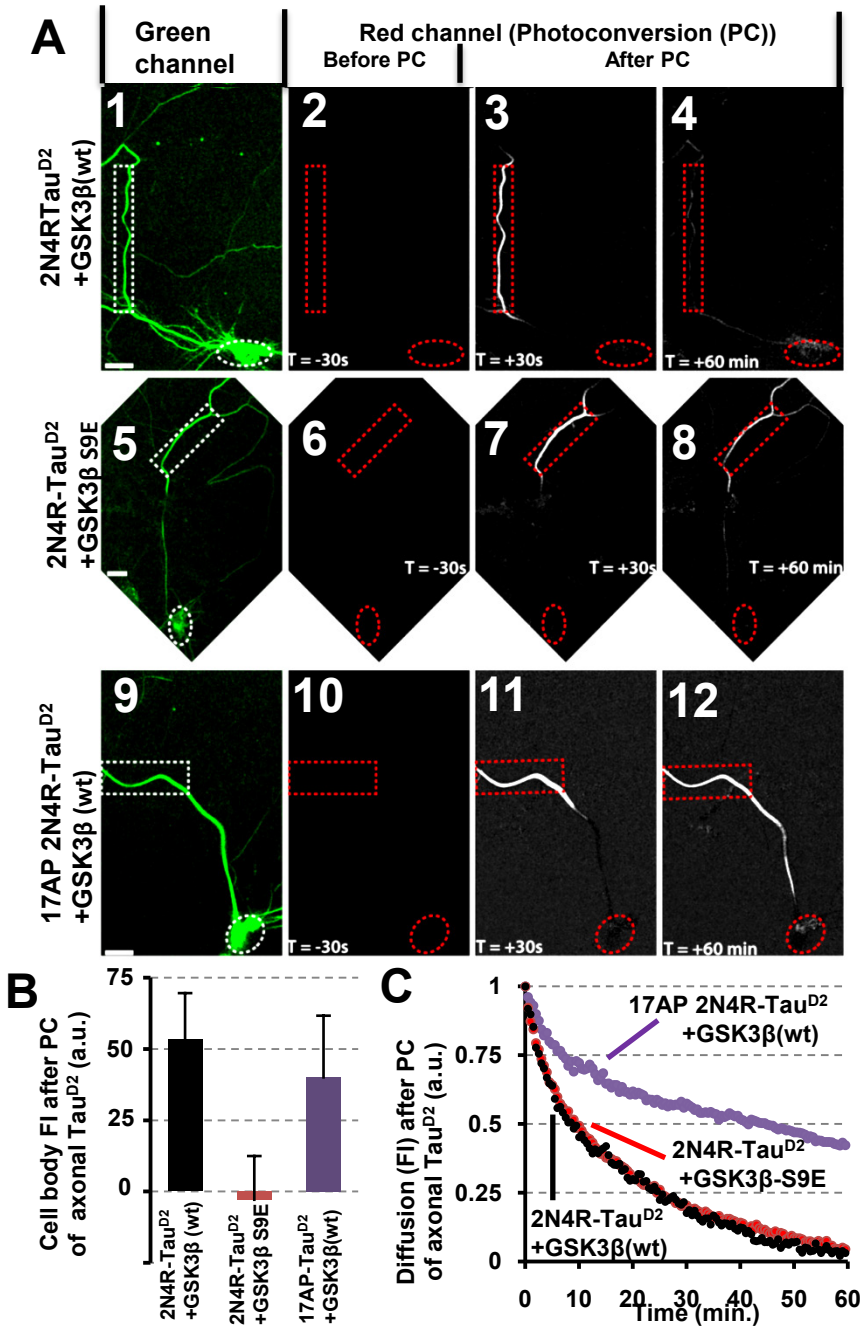

A
Green channel
Red channel (Photoconversion (PC))
Before PC
After PC
2N4RTauD2 +GSK3β(wt)
2N4R-TauD2 +GSK3β S9E
17AP 2N4R-TauD2 +GSK3β (wt)
T = -30s
T = +30s
T = +60 min
B
Cell body FI after PC of axonal TauD2 (a.u.)
2N4R-TauD2 +GSK3β (wt)
2N4R-TauD2 +GSK3β S9E
17AP-TauD2 +GSK3β(wt)
C
Diffusion (FI) after PC of axonal TauD2 (a.u.)
17AP 2N4R-TauD2 +GSK3β(wt)
2N4R-TauD2 +GSK3β-S9E
2N4R-TauD2 +GSK3β(wt)
Time (min.)

Figure 4

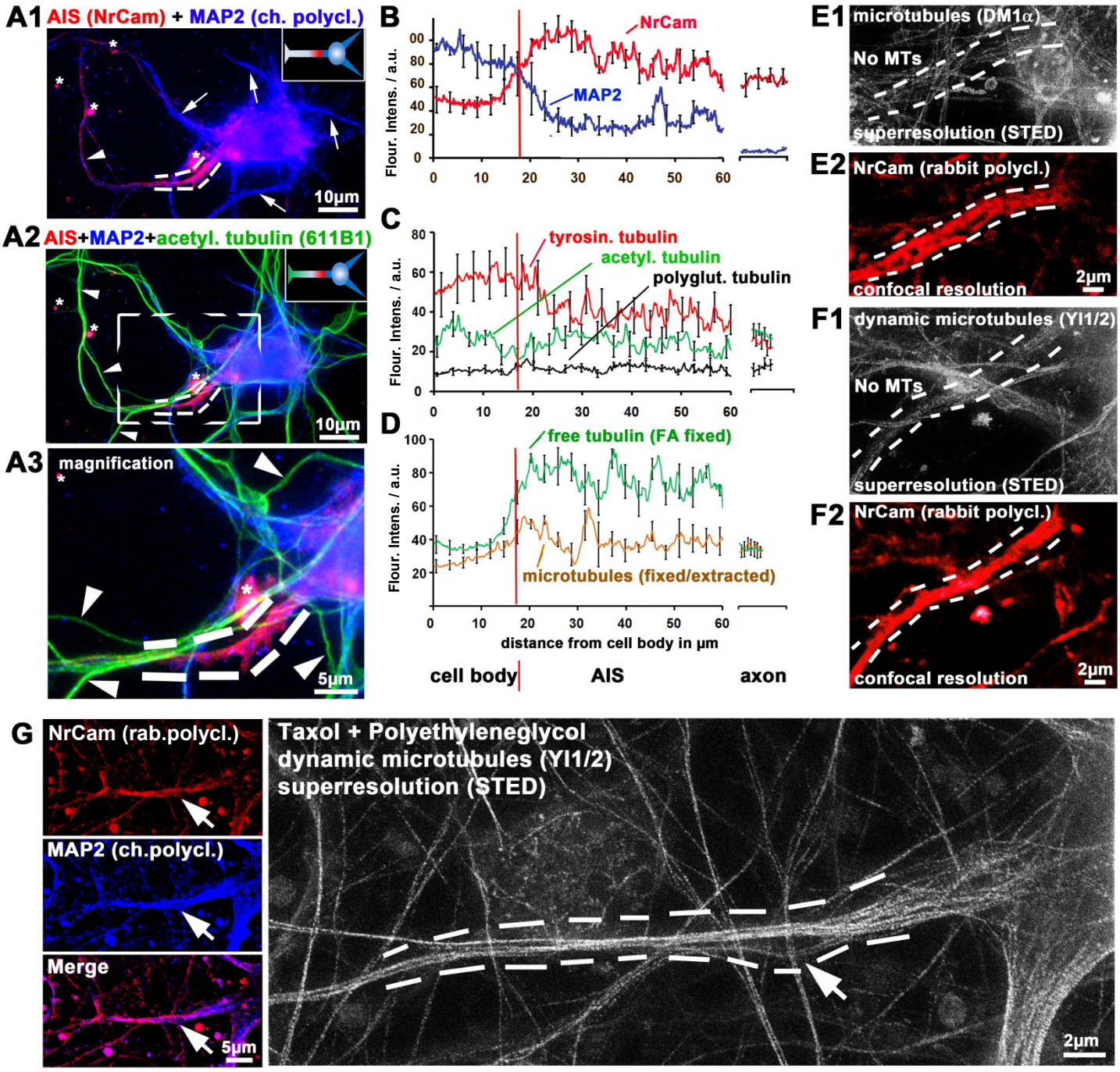

A1
AIS (NrCam) + MAP2 (ch. polycl.)
10µm
A2
AIS+MAP2+acetyl. tubulin (611B1)
10µm
A3
magnification
5µm
B
NrCam
MAP2
Flour. Intens. / a.u.
C
tyrosin. tubulin
acetyl. tubulin
polyglut. tubulin
Flour. Intens. / a.u.
D
free tubulin (FA fixed)
microtubules (fixed/extracted)
Flour. Intens. / a.u.
distance from cell body in µm
cell body
AIS
axon
E1
microtubules (DM1α)
No MTs
superresolution (STED)
E2
NrCam (rabbit polycl.)
confocal resolution
2µm
F1
dynamic microtubules (YI1/2)
No MTs
superresolution (STED)
F2
NrCam (rabbit polycl.)
confocal resolution
2µm
G
NrCam (rab.polycl.)
MAP2 (ch.polycl.)
Merge
5µm
Taxol + Polyethyleneglycol
dynamic microtubules (YI1/2)
superresolution (STED)
2µm

Figure 5

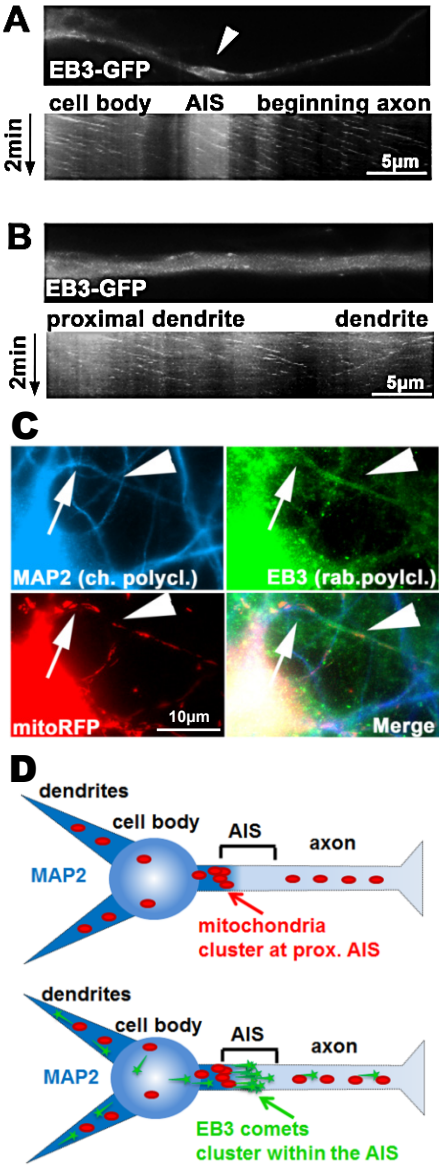
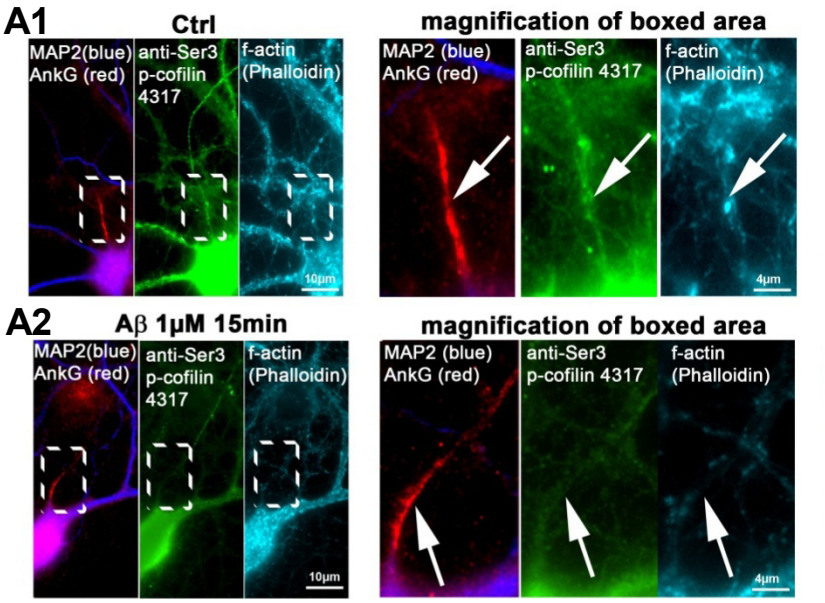

A
EB3-GFP
cell body
AIS
beginning axon
2min
5µm
B
EB3-GFP
proximal dendrite
dendrite
2min
5µm
C
MAP2 (ch. polycl.)
EB3 (rab.poylcl.)
mitoRFP
10µm
Merge
D
dendrites
cell body
AIS
axon
MAP2
mitochondria
cluster at prox. AIS
dendrites
cell body
AIS
axon
MAP2
EB3 comets
cluster within the AIS

Figure 6


A1
Ctrl
MAP2(blue)
AnkG (red)
anti-Ser3
p-cofilin
4317
f-actin
(Phalloidin)
10μm
magnification of boxed area
MAP2 (blue)
AnkG (red)
anti-Ser3
p-cofilin 4317
f-actin
(Phalloidin)
4μm
A2
Aβ 1μM 15min
MAP2(blue)
AnkG (red)
anti-Ser3
p-cofilin
4317
f-actin
(Phalloidin)
10μm
magnification of boxed area
MAP2 (blue)
AnkG (red)
anti-Ser3
p-cofilin 4317
f-actin
(Phalloidin)
4μm

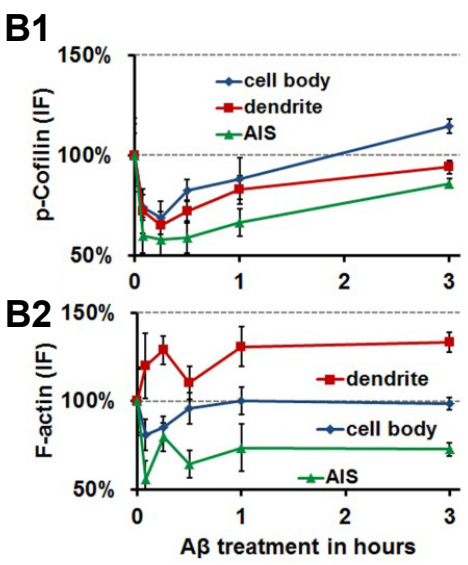
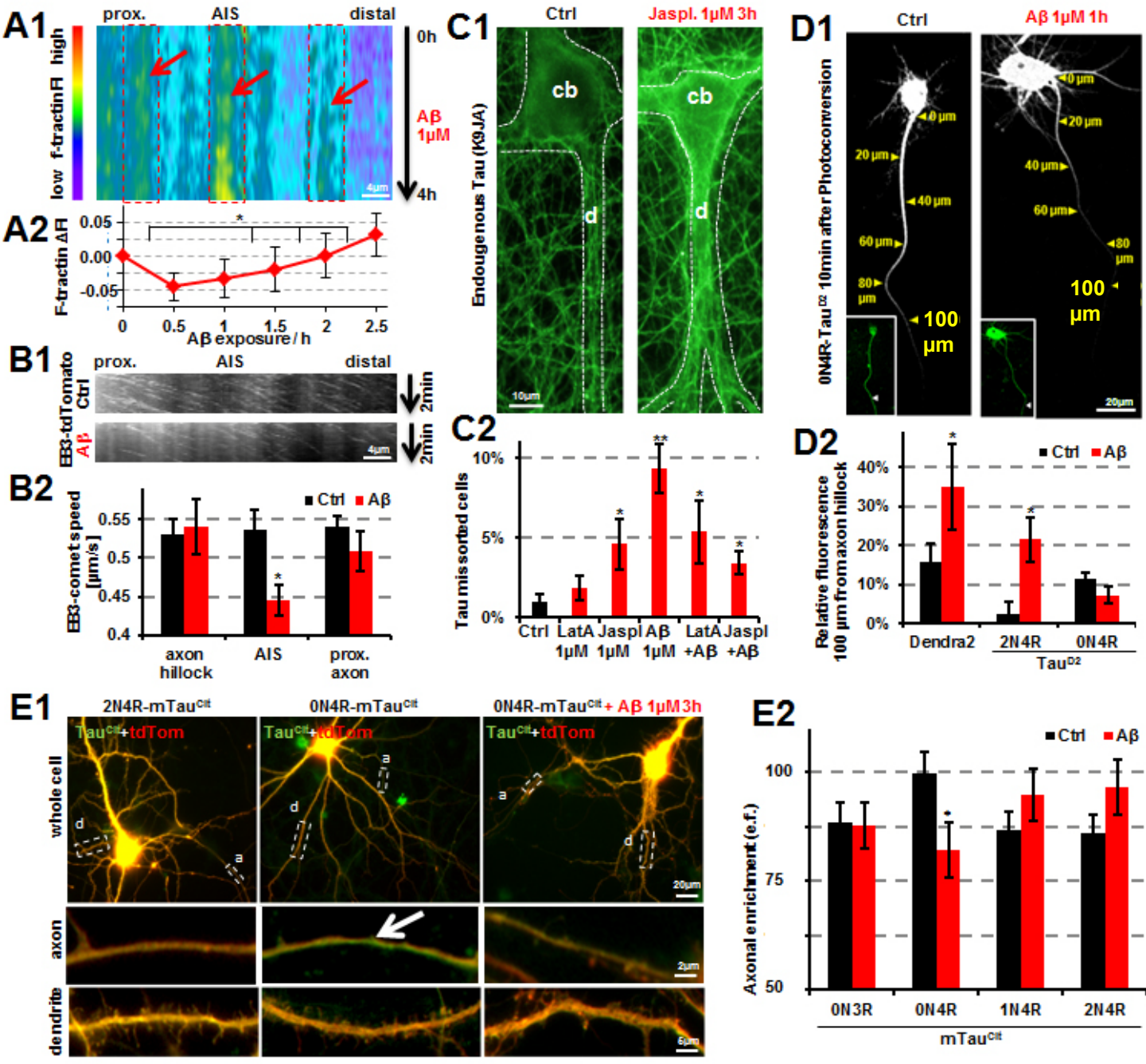

B1
150%
100%
50%
p-Cofilin (IF)
cell body
dendrite
AIS
0
1
2
3
B2
150%
100%
50%
F-actin (IF)
dendrite
cell body
AIS
0
1
2
3
Aβ treatment in hours

Figure 7


A1
prox.
AIS
distal
high
low
f-tractin FR
0h
Aβ 1μM
4h
4μm
A2
F-tractin ΔFR
0.05
0.00
-0.05
*
0
0.5
1
1.5
2
2.5
Aβ exposure / h
B1
prox.
AIS
distal
EB3-tdTomato
Ctrl
Aβ
2min
2min
4μm
B2
EB3-comets speed [μm/s]
Ctrl
Aβ
0.55
0.5
0.45
0.4
*
axon hillock
AIS
prox. axon
C1
Ctrl
Jaspl. 1μM 3h
Endougenous Tau (K9JA)
cb
d
10μm
C2
Tau missorted cells
10%
5%
0%
*
**
Ctrl
LatA 1μM
Jaspl 1μM
Aβ 1μM
LatA +Aβ
Jaspl +Aβ
D1
Ctrl
Aβ 1μM 1h
0N4R-Tau D2 10min after Photoconversion
0 μm
20 μm
40 μm
60 μm
80 μm
100 μm
20μm
D2
Relative fluorescence 100 μm from axon hillock
Ctrl
Aβ
40%
30%
20%
10%
0%
*
Dendra2
2N4R
0N4R
Tau D2
E1
2N4R-mTau Cit
0N4R-mTau Cit
0N4R-mTau Cit + Aβ 1μM 3h
Tau Cit +tdTom
whole cell
axon
dendrite
a
d
20μm
2μm
6μm
E2
Axonal enrichment (e.f.)
Ctrl
Aβ
100
75
50
*
0N3R
0N4R
1N4R
2N4R
mTau Cit

Axodendritic sorting and pathological missorting of Tau is isoform specific and determined by axon initial segment architecture

Hans Zempel, Frank Dennissen, Yatender Kumar, Julia Luedtke, Jacek Biernat, Eva-Maria Mandelkow and Eckhard Mandelkow

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