Appendix Material:

Table of Appendix content:

Name	Title	Page
Appendix Figure S1	Disorder and low complexity domain (LCD) predictions for tau441 protei	2
Appendix Figure S2	GFP-tau441 droplets do not co-localize with endosomes or the ER	4
Appendix Figure S3	Atomic force microscopy of p-tau441 droplets	5
Appendix Supplementary Methods	Estimating the tau concentration in neurons of human cortex	6-8
Appendix Table S1	Measurements and calculations for human brain tau concentration – Sections sizes & Volume	9
Appendix Table S2	Measurements and calculations for human brain tau concentration – Stereological neuron count & brain tissue wet weight	10
Appendix Table S2	Measurements and calculations for human brain tau concentration – Tau ELISA & Tau per cell/tissue volume	11
Appendix Table S4	List of antibodies	12

A Disorder prediction for tau (2N4R, 441 aa) by disEMBL

Disorder by Loops/Coils definition

```
maeprqefev mEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPG
SETSDAKSTP TAEDVTAPLV DEGAPGKQAA AQPHTEIPEG TTAEEAGIGD TPSLEDEAAg
hvtqarmVSK SKDGTGSDDK KAKGADGKTK IATPRGAAPP GQKGQANATR IPAKTPPAPK
TPPSSGEPPK SGDRSGYSSP GSPGTPGSRS RTPSLPTPPT REPKKVAVVR TPPKSPSSAK
SRLQTAPVPM PDLKNVKSKI GSTENLKHQP GGGKVqiink klDLSNVQSK CGSKDNIKHV
PGGGSVQIVY KPVDLSKVTS KCGSLGNIHH KPGGGQVEVK SEKLDFKDRV QSKIGSLDNI
THVPGGGNKK IETHKltfre naKAKTDHGA EIVYKSPVVS GDTSPRHLSN VSSTGSIDMV
DSPQLATlad evsaslakqg l
```

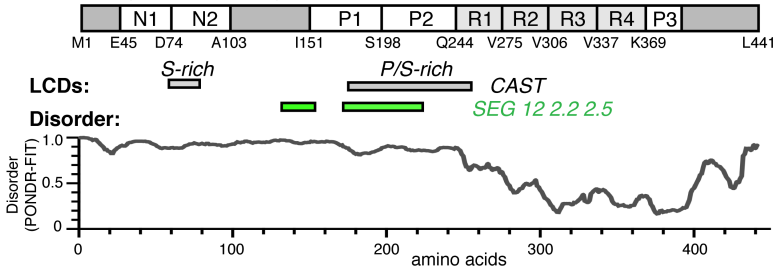
Disorder by Hot-loops definition

```
maeprqefev medhagtygl gdrkdqggyt mhqdqegdtd aglkesplqT PTEDGSEEPG
SETSDAKSTP TAedvtaplv degapgkqaa aqphteipeg ttaeeagigd tpsledeaag
hvtqARMVSK SKDGTGSDDK KAKGADGKTK IATPRGAAPP GQKGQANATR IPAKTPPAPK
TPPSSGEPPK SGDRsgyssP GSPGTPGSRS RTPSLPTPPT REPKKVAVVR TPPKSPSSAK
SRLQTAPVPM PDLknvkski gSTENLKHQP GGGKvqiink kldlsnvqsk cgSKDNIKHV
PGGGSVqivy kpvdlskvts kcgsLGNIHH KPGGGQVEVK Sekldfkdrv QSKIGSLDNI
THVPGGGNKK IETHkltfRE NAKAKTDHGA EIVYKSPVVS GDTSPRHlsn vsstgsidmv
dspqlatlad evsaslakqg l
```

Disorder by Remark-465 definition

```
MAEPRQEFEv medhagtygl gdrkdqggyt mhqdqegdtd aglkesPLQT PTEDGSEEPG
SETSDAKSTP TAedvtaplv degaPGKQAA AQPHteipeg ttaeeagigd tpsledeAAG
HVTQARMVSK SKDGTGSDDK KAKGADGKTk iatprGAAPP GQKGQANATr ipaKTPPAPK
TPPSSGEPPK SGDRSGYSSP GSPGTPGSRS RTPSLPTPPT REPKKVAVVR TPPKSPSSAK
SRLQTapvpm pdlknvkski gstenlkhqp gggkvqiink kldlsnvqsk cgskdnikhv
pgggsvqivy kpvdlskvts kcgslgnihh kpgggqvevk sekldfkdrv qskigsldni
thvpgggnkk iethkltfre nakaktdhga eivykspvvS GDTSPRHLSN vsstgsidmv
dspqlatlad evsaslakqg l
```

B Low complexity domain (LCD) predictions for tau (2N4R, 441 aa)



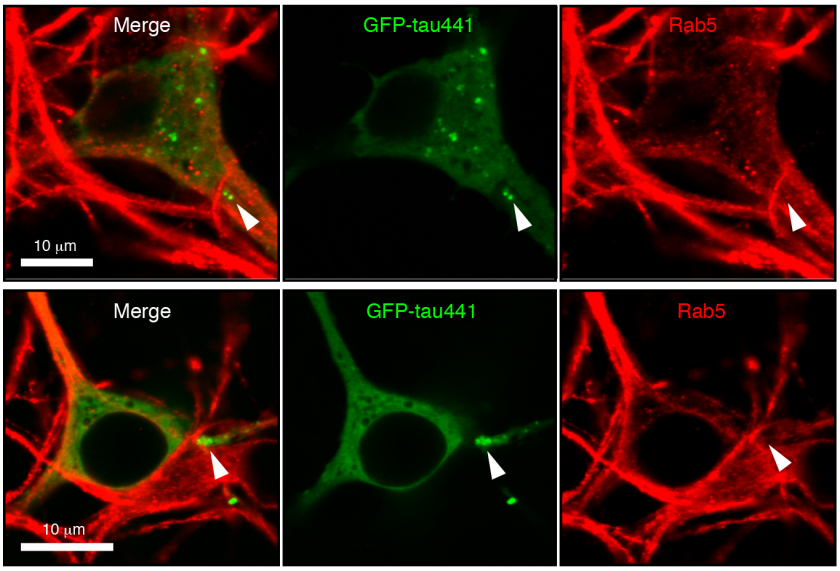
Appendix Figure S1 - Disorder and low complexity domain (LCD) predictions for tau441 protein.

Tau is an intrinsically unfolded protein with some low propensity secondary structure predictions (Mukrasch *et al*, 2009), and its high amino acid and charge complexity makes it extremely soluble. Here predicted LCDs are based on high content of prolines and serines in certain areas.

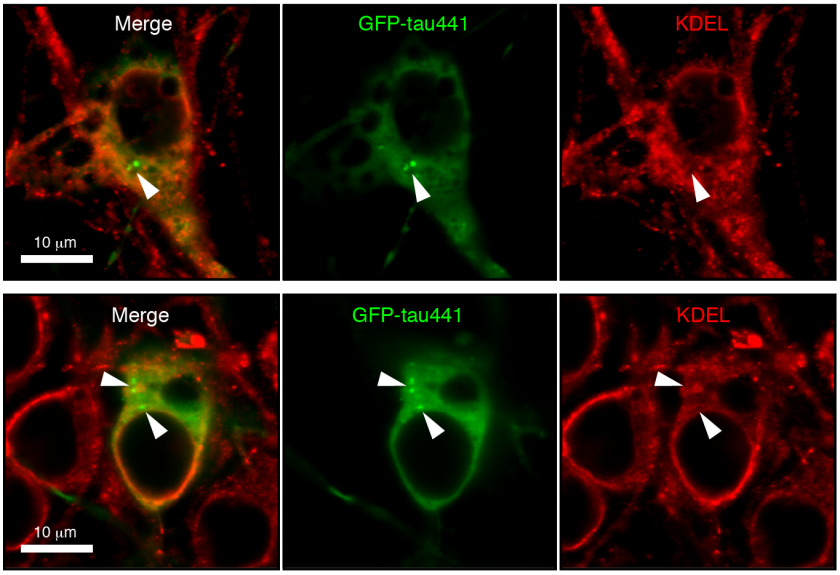
A Disordered region in tau441 (2N4R, 441 aa) following different prediction definitions are shown on a single residue level. Predictions were run using disEMBL (http://dis.embl.de/), disordered residues are highlighted in color.

B Prediction of low complexity domains (LCD) in tau441 were run using the predictors CAST (http://dis.embl.de/) and SEG (http://mendel.imp.ac.at/METHODS/seg.server.html) and the position of putative LCDs (=low complexity in the primary aa sequence) in the sequence of tau441 are displayed indicated as bars (CAST= grey bars; SEG=green bars) aligned to tau441 sequence and disorder prediction by PONDR (http://pondr.com). Note: the predicted LCD in P1/P2 results from high concentration of serines and prolines but does not necessarily indicate common LCD behavior; there is no prion-like LCD in tau441 as for most other LLPS proteins.

A **Non**-colocalization of GFP-tau441 droplets with endosomes (Rab5)

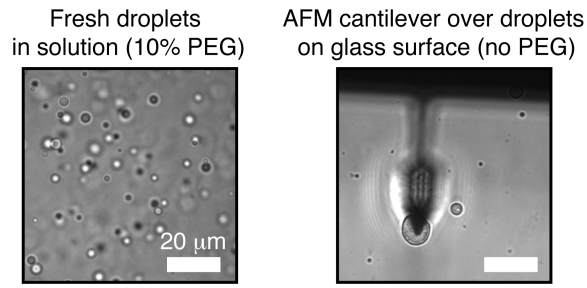


B **Non**-colocalization of GFP-tau441 droplets with the ER (KDEL)



Appendix Figure S2 - GFP-tau441 droplets do not co-localize with endosomes or the ER.

A, B In primary cortical mouse neurons, droplet-like GFP-tau441 (white arrow heads) did not colocalize with (A) endosomes (Rab5) or (B) the endoplasmic reticulum (ER) marker KDEL.



Appendix Figure S3 - Atomic force microscopy of p-tau441 droplets.

p-tau441 droplets were prepared in presence of 10% PEG (left) and deposited on a flat clean glass surface and incubated for 2-3 h at room temperature to allow stable adsorption to the surface. Afterwards, the supernatant was carefully exchanged against buffer without PEG (20 mM HEPES pH 7.4, 50 mM NaCl) to remove the excess droplets. The AFM head was placed on top of the sample and the cantilever positioned above the stably adsorbed droplets to start the AFM scanning (right). AFM topographies were then recorded at scanning forces of 250-300 pN (see Figure 4F).

Appendix Supplementary Methods:

Estimating the tau concentration in neurons of human cortex

We counted the number of neurons in a block of human frontal lobe grey matter of known volume by stereology, and measured the amount of tau (grams) in adjacent brain tissue of of known volume by ELISA. Since we expect that tau is not uniformly distributed in neurons we used these measurements to estimate the range of likely intracellular concentrations: the minimum tau concentration assumed an even distribution of tau in the entire cortical volume (=maximum volume occupied). The maximum tau concentration (=minimum volume) was calculated by normalizing the tau content in human frontal lobe grey matter (determined by ELISA) to the number of neurons and an average soma volume (=approximated as sphere with 20 μm diameter). Raw data is presented in Table S1.

1. Amount (grams) tau per neuron in human frontal lobe cortex:

Frozen tissue blocks (adjacent to those used for tau ELISA (2)) were fixed for 48 h in 4% paraformaldehyde/PBS and then cryo-protected in sucrose overnight. For each of the human cases (2x AD, 1x Control), a small (≈1 cm^3) tissue block of the frontal lobe grey matter was weighed and then cut into 50 μm thick sections. One hundred sections per block were collected as floating sections, and the remainder of the tissue, as well as any discarded tissue, was weighed to determine the total weight of cut slices.

We then to label neurons in the mounted sections by immunohistochemistry using an anti-NeuN antibody was performed to label neurons. In brief, tissue was washed with TBS, then in 0.03% hydrogen peroxide for 10 minutes, then blocked an permeabilized for 1 h with 3% normal goat serum/TBS containing 0.25% Triton-X (TBS-X), and then incubated in mouse anti-NeuN (Millipore, 1:1000) in the blocking buffer overnight. The following day, sections were washed in TBS, then incubated in biotinylated anti-mouse secondary followed by ABC compound (Vectorlabs) and DAB with 0.15% nickel chloride (Sigma-Aldrich). Sections were mounted, dehydrated in an ascending ethanol series followed by xylene, and then coverslipped with Cytoseal XYL (Thermofisher).

Stereological data collection:

Neuronal counts were obtained by stereology (CAST software, Olympus): NeuN-labeled cell bodies within Grey matter were counted in 1% of three cortical sections, randomly sampled from the cut blocks. Neuron counts per section were multiplied by 100 to estimate the number of neurons per 100% section.

(1) The total number of neurons per volume (mm^3) was calculated as:

[Neuron #/mm^3 sections] = # of neurons per section * Grey matter volume

with Grey matter volume (mm^3) = [(analyzed Grey matter area (μm^2) x 50 μm section thickness) $*10^{-9}$]

(2) The total weight of sectioned grey matter was calculated as:

[grams of Grey matter] = % grey matter area per section * weight of the cut tissue.

(3) The number of neurons per gram of Grey matter was then calculated as followed:

[Neuron #/gram Grey matter] = [Neuron #/mm^3 sections] * [(Grey Matter Volume (mm^3) per section * 100 sections)/ [grams of Grey matter]

Human tau ELISA:

Finally, to determine the amount of tau per neuron, we performed an anti-human tau ELISA (Thermoscientific) on known amounts of Grey matter from the same brain areas (tissue cut adjacent to the blocks for stereology):

[Grams of tau per neuron] = tau per gram tissue / Neurons per gram tissue

Results (mean±s.d. from three separate tissue preps per sample, see Appendix Supplementary Tables S1, S2, S3):

Control: $1.61 \times 10^{-12} \pm 0.3$ g tau per neuron = 1.61 ± 0.3 picogram (pg) tau per neuron

AD #1: $1.06 \times 10^{-12} \pm 0.6$ g tau per neuron = 1.06 ± 0.6 pg tau per neuron

AD #2: $1.29 \times 10^{-12} \pm 0.8$ g tau per neuron = 1.29 ± 0.8 pg tau per neuron

[Grams of tau per neuron] (Mean±s.d.) = **1.32** ± **1.04 pg tau per neuron** **(4)**

2. Estimating the volume occupied by a neuron in the human frontal lobe cortex:

The highest tau concentration would occur if tau is restricted to only the neuronal cell body. We estimated the minimum volume of a neuronal cell bodies assuming a spherical shape of a pyramidial neuron that has a soma diameter of ≈20 μm:

$V_{cell\ body} = 4/3*\pi*(d/2)^3 = 4/3*\pi*(10)^3\ \mu m^3 = 4.19*10^{-6}\ \mu l$

With 1.32 ± 1.04 pg tau per neuron **(4)** and a molecular weight of full-length tau of ≈46 kDa (=$46*10^3$ g/mol), this gives an average tau concentration in the soma:

$[tau]_{soma}$ = 6.84±0.42 μM (5)

The lowest tau concentration would occur if tau would be distributed evenly through the entire cortical volume. In this case, we can calculate the mean cortical concentration tau of as:

$[tau]_{cortex}$ full= (tau/cortex weight) / (cortex volume/cortex weight) * (molecular weight of full-length tau)

$[tau]_{cortex\ full}$ = 0.42±0.17 μM

This estimate neglects the fact that ≈25% of cortical tissue volume is occupied by the vascular system and the extracellular space; taking this into account increases the tau concentration in the cortical volume to:

$[tau]_{cortex}$ = 1.68±0.68 μM (6)

Appendix Table S1 - Measurements and calculations for human brain tau concentration – Sections sizes & Volume

		Area measured from sections							
Human Brain Case #	Sample #	Gray Matter total area (um2)	White Matter Area (um2)	Total Area of Section (um2)	% Gray Matter	% White Matter	Cut section thickness (um)	Gray matter Volume per section (mm3)	Est. Vol Grey Matter Cut Block (mm3)
2012-1	Area 1	21403000	37267000	58670000	36.48031362	63.51968638	50	1.07015	145.36
	Area 2	27885000	40365000	68250000	40.85714286	59.14285714	50	1.39425	
	Area 3	37928000	24662000	62590000	60.59753954	39.40246046	50	1.8964	
2012-2	Area 1	58100000	34460000	92560000	62.77009507	37.22990493	50	2.905	307.9333333
	Area 2	64410000	28700000	93110000	69.17624315	30.82375685	50	3.2205	
	Area 3	62250000	25550000	87800000	70.89977221	29.10022779	50	3.1125	
2012-3	Area 1	96670000	38430000	135100000	71.55440415	28.44559585	50	4.8335	454.05
	Area 2	99220000	36980000	136200000	72.84875184	27.15124816	50	4.961	
	Area 3	76540000	41860000	118400000	64.64527027	35.35472973	50	3.827	
2021-1	Area 1	128200000	33700000	161900000	79.1846819	20.8153181	50	6.41	615.6833333
	Area 2	118900000	3900000	122800000	96.82410423	3.175895765	50	5.945	
	Area 3	122310000	42990000	165300000	73.99274047	26.00725953	50	6.1155	
2021-2	Area 1	56142000	40908000	97050000	57.84853168	42.15146832	50	2.8071	277.905
	Area 2	52320000	49280000	101600000	51.49606299	48.50393701	50	2.616	
	Area 3	58281000	32459000	90740000	64.22856513	35.77143487	50	2.91405	
2021-3	Area 1	98720000	44580000	143300000	68.89043964	31.10956036	50	4.936	476.6833333
	Area 2	95030000	43770000	138800000	68.46541787	31.53458213	50	4.7515	
	Area 3	92260000	37640000	129900000	71.02386451	28.97613549	50	4.613	
2048-1	Area 1	71640000	33160000	104800000	68.35877863	31.64122137	50	3.582	388.2
	Area 2	66270000	35330000	101600000	65.22637795	34.77362205	50	3.3135	
	Area 3	95010000	47690000	142700000	66.58023826	33.41976174	50	4.7505	
2048-2	Area 1	86070000	27630000	113700000	75.69920844	24.30079156	50	4.3035	443.8833333
	Area 2	102000000	40800000	142800000	71.42857143	28.57142857	50	5.1	
	Area 3	78260000	11090000	89350000	87.58813654	12.41186346	50	3.913	
2048-3	Area 1	84060000	20840000	104900000	80.13346044	19.86653956	50	4.203	397.2725
	Area 2	74849000	18401000	93250000	80.26702413	19.73297587	50	3.74245	

Appendix Table S2 - Measurements and calculations for human brain tau concentration – Stereological neuron count & brain tissue wet weight

Neuron count and wet weight of tissue sections

Human Brain Case #	Sample #	Neurons per section	Total Weight of Cut Slices (g)	Total Weight of Grey Matter in Cut Slices (g)	Neurons/ Volume Grey Matter (mm3)	Avg. Neurons/Volume Grey Matter (mm3)	mm3/g	Neurons/g
2012-1	Area 1	15300	0.626	0.287824358	14297.06116	14496.31477	505.0302234	7321077.084
	Area 2	22100			15850.81585			
	Area 3	25300			13341.06729			
2012-2	Area 1	49000	0.643	0.43476683	16867.46988	17261.7712	708.2723705	12226035.6
	Area 2	57200			17761.2172			
	Area 3	53400			17156.62651			
2012-3	Area 1	69200	0.584	0.406947603	14316.7477	13408.31537	1115.745606	14960268.95
	Area 2	65400			13182.82604			
	Area 3	48700			12725.37235			
2021-1	Area 1	100300	0.288	0.240001466	15647.4259	17015.36514	2565.331557	43650053.16
	Area 2	110900			18654.33137			
	Area 3	102400			16744.33816			
2021-2	Area 1	49300	0.537	0.310695956	17562.6091	17220.68611	894.4596625	15403209.08
	Area 2	43600			16666.66667			
	Area 3	50800			17432.78255			
2021-3	Area 1	72400	0.733	0.509141121	14667.74716	14085.05205	936.2499195	13187128.85
	Area 2	63100			13280.01684			
	Area 3	66000			14307.39215			
2048-1	Area 1	41400	0.676	0.451039356	11557.78894	12129.9044	860.6787734	10439951.24
	Area 2	40500			12222.72522			
	Area 3	59900			12609.19903			
2048-2	Area 1	61600	0.546	0.427182968	14313.93052	13489.88359	1039.094174	14017259.45
	Area 2	57800			11333.33333			
	Area 3	58000			14822.38692			
2048-3	Area 1	68000	0.663	0.531727606	16178.91982	16252.56162	747.1353664	12142863.58
	Area 2	61100			16326.20342			

Appendix Table S3: Measurements and calculations for human brain tau concentration – Tau ELISA & Tau per cell/tissue volume

Tau concentration measured by ELISA							Tau ELISA on homogenate from 0.2 g Gray matter					
Human Brain Case #	**ELISA huTau ug/g *****	**tau ug/neuron**	**spherical cell body vol /ul**	**tau in cell body ug/ul**	**tau conc in cell body (uM)**	**tau conc per tissue volume (uM)**	**Human Brain Case #**	ABS	tau CONC	Dilution factor	huTau ng/ml	**ELISA huTau ug/g *****
2012-1	14.18152041	1.93708E-06	4.18667E-06	0.462678618	10.05823084	0.61044648	2012-1	0.385467672	0.284	1000	2836.304024	**14.18152041**
							2012-2	0.519826639	0.394	1000	3941.9572	**19.709786**
							2012-3	0.504814249	0.382	1000	3818.41877	**19.09209385**
2012-2	19.709786	1.61212E-06	4.18667E-06	0.385059523	8.370859186	0.604955984	2021-1	0.440198076	0.329	1000	3286.685942	**16.43342971**
							2021-2	0.512047672	0.388	1000	3877.943321	**19.3897166**
							2021-3	0.538699577	0.410	1000	4097.26446	**20.4863223**
2012-3	19.09209385	1.27619E-06	4.18667E-06	0.304821625	6.626557075	0.371989382	2048-1	0.602914321	0.463	1000	4625.693883	**23.12846942**
							2048-2	0.311590086	0.223	1000	2228.358181	**11.14179091**
							2048-3	0.286627873	0.202	1000	2022.94168	**10.1147084**
2021-1	16.43342971	3.76481E-07	4.18667E-06	0.089923883	1.954867013	0.139260156						
2021-2	19.3897166	1.25881E-06	4.18667E-06	0.300671225	6.536330968	0.471251635						
2021-3	20.4863223	1.55351E-06	4.18667E-06	0.371061048	8.066544519	0.475679435						
2048-1	23.12846942	2.21538E-06	4.18667E-06	0.529151508	11.50329366	0.584181728						
2048-2	11.14179091	7.94862E-07	4.18667E-06	0.189855642	4.127296561	0.233099994						
2048-3	10.1147084	8.32976E-07	4.18667E-06	0.198959127	4.325198409	0.294304051						
					6.841019803	**0.420574316**	Mean	Tau concentration (µM)				
					3.033902879	**0.171394699**	S.D.					

Appendix Table S4 – List of antibodies

antigen	name	source species	source	number/ ID
tau antibodies:				
human total tau	Tau13	mouse	Biolegend	#MMS-520R
human total tau	KJ9A	rabbit	DAKO	A0024
pT181		mouse	Thermofisher	#MN1050
pS199		rabbit	Thermofisher	#710124
pS199		rabbit	Cell SignalingTechnology	#29957
pS202		rabbit	Cell SignalingTechnology	#39357
pT205		rabbit	Thermofisher	#44-738G
pT212		rabbit	Thermofisher	#44-740G
pS214		rabbit	Abcam	#ab170892
pT231		rabbit	Thermofisher	#710126
pT231		rabbit	Abcam	#ab151559
pS235		rabbit	GeneTex	#GTX86624
pS262		rabbit	Thermofisher	#44-750G
pS324		rabbit	Abcam	#ab109401
pS396		rabbit	Abcam	#ab109390
pS404		rabbit	Cell SignalingTechnology	#20194
pS409		rabbit	Thermofisher	#44-760G
pS422		rabbit	Abcam	#ab79415
pS202/S205	CP12	mouse	Peter Davis Lab	n.a.
pS202/S205	AT8	mouse	Thermofisher	#MN1020
pS394/pS404	PHF1	mouse	Peter Davis Lab	n.a.
pS262/pS356	12e8	mouse	Elan Pharmaceuticals	n.a.
Organelle markers				
Lamp1		rabbit	Abcam	ab24170
Rab5		rabbit	SantaCruz	sc-46692
KDEL		rabbit	Abcam	ab176333