

Supplementary Text

Our data suggests that α -IgLON5 AABs induce pathogenic effects in the brain through neuroinflammation, neuronal physiology, and Tau. This combination of insults likely determines disease progression and clinical manifestation of individual anti-IgLON5 disease patients (22, 75, 76). We find that all polyclonal α -IgLON5 AAB pools have a similar IgG-subclass composition and similar amounts of IgM. The subclass composition of total plasma IgGs of these patients could still be similar to previous reports though (mostly subclasses IgG1 and IgG4) (19, 22). Notably, the IgG subclass composition of α -IgLON5 AABs can have large effects on neuroinflammation and related neurotoxicity, and therefore could also influence Tau pathology in anti-IgLON5 disease patient brain. For the acute effects of α -IgLON5 AABs on neuronal activity and Tau missorting, detected herein in cultured neurons, the influence of IgG subclass composition may be of less importance.

Parenchyma concentration and distribution of cerebroventricularly delivered antibodies in our mouse model are different from that in anti-IgLON5 disease patients. Hence, the brain regions affected by the increase in phosphorylated Tau differ between the animal model and human brains. For example, we observe Tau phosphorylation selectively in the hippocampus, a region of high α -IgLON5 AAB concentration in our model. In these mice, accumulation of Tau-pS396/pS404 occurred in somata of DG granule cells and their projections onto CA3, important structures for learning and memory, and in commissural fibres in corpus callosum, output structures of CA3. Similar hippocampal connections were recently suggested to be functionally affected by intraventricularly delivered α -IgLON5 AABs (51, 52), supporting our observations, and are also affected by Tau in AD (77, 78). Notably, upon two weeks of AAB delivery, we did not detect neurotoxicity or Tau aggregation in wildtype mice. We suspect that long-term (e.g., 1-6 months) or repeated cerebroventricular infusion of non-toxic AAB doses may be needed to induce full Tau pathology and neurodegeneration.

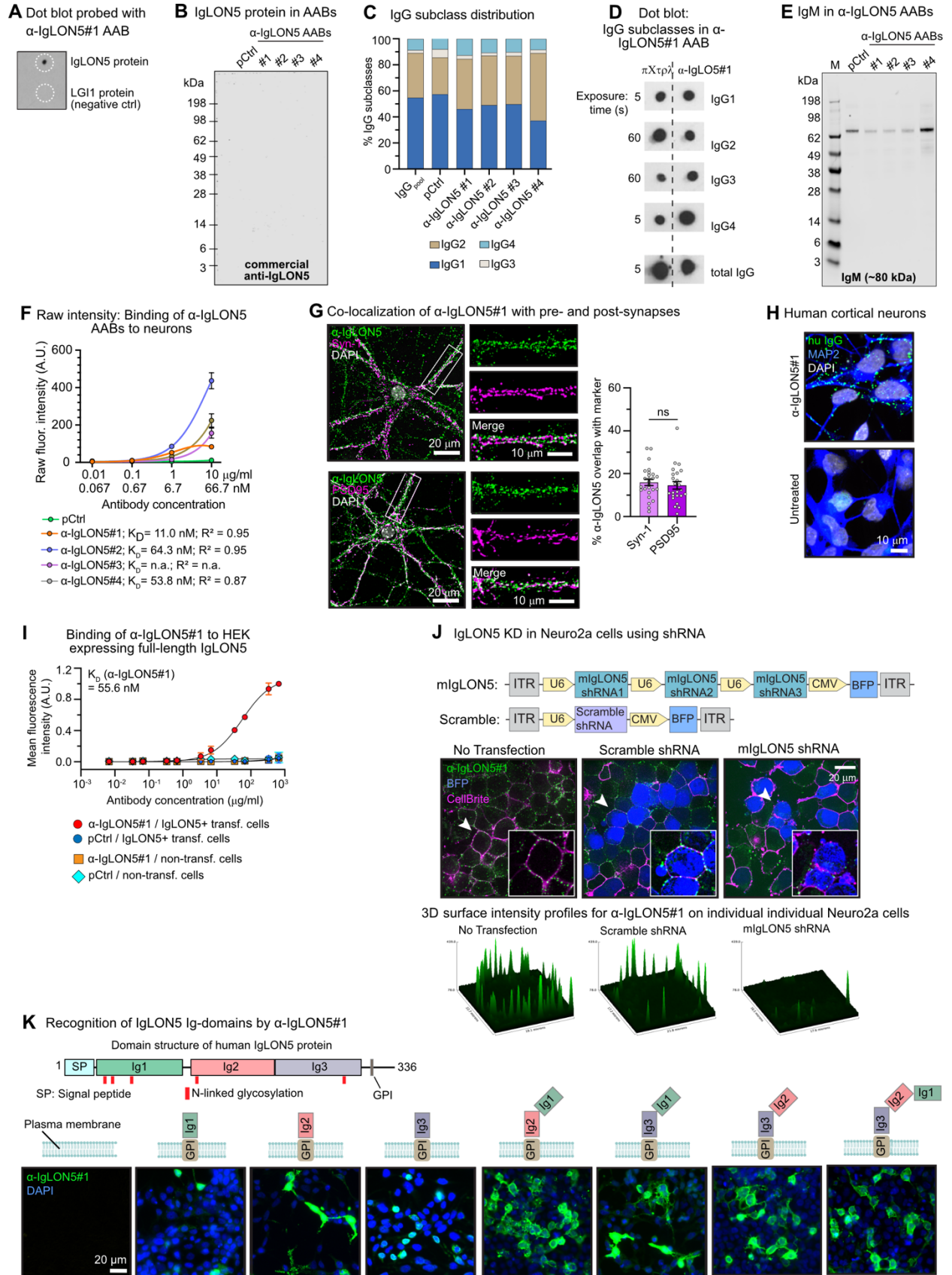
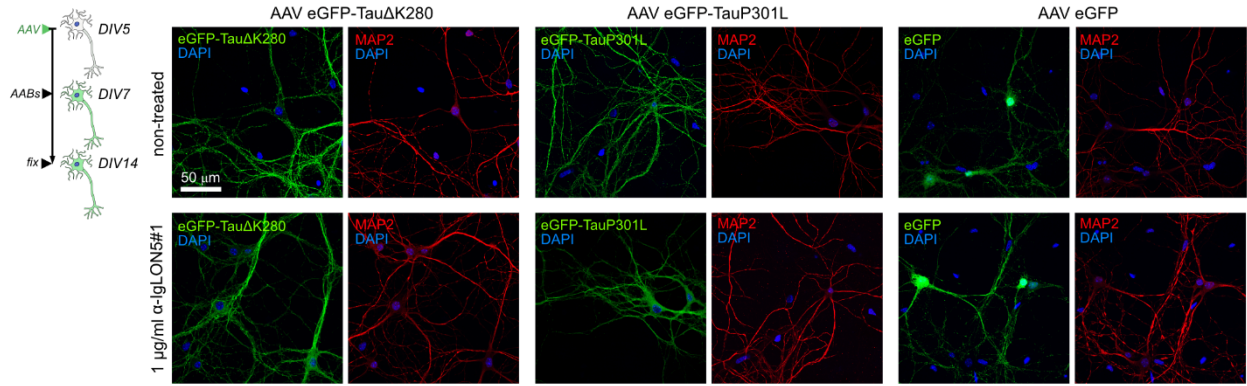


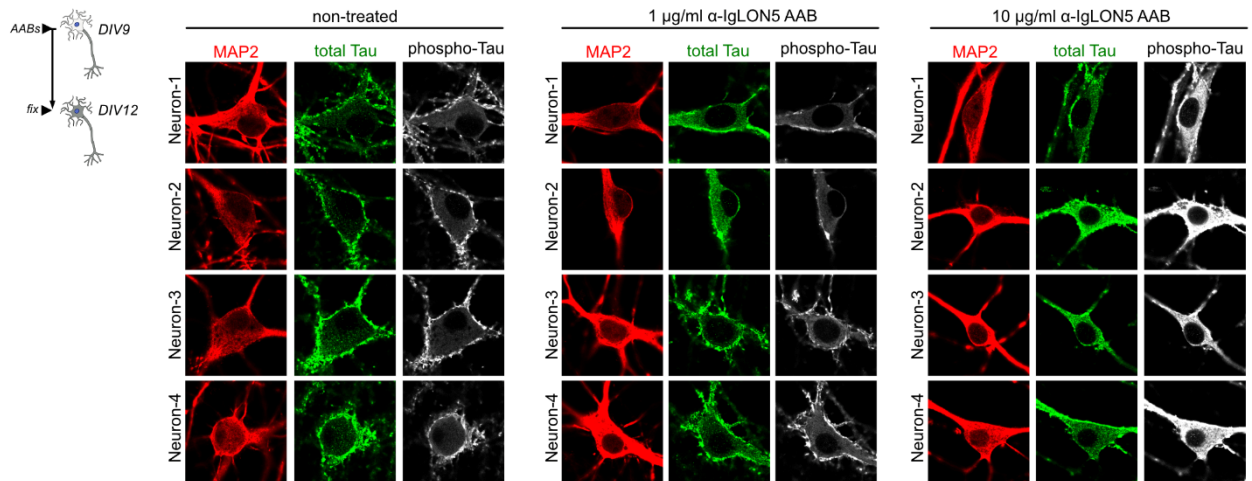
Fig. S1. Supplementary Figure S1. α -IgLON5 AABs characterization.

(A) Dot blot of recombinant IgLON5 and LGI (negative ctrl) protein shows specific reactivity of AAB α -IgLON5#1 against IgLON5 protein. (B) Western blot, probed with commercial anti-IgLON5 antibody, shows no IgLON5 protein in AAB preparations. (C) ELISA-based quantification of IgG subclasses in α -IgLON5 AAB and pCtrl preparations and, for comparison, in a commercial human serum IgG pool (IgG_{pool}). (D) Dot blot for IgG subclasses (IgG₁, IgG₂, IgG₃ and IgG₄) in α -IgLON5#1 and pCtrl. Note, different exposure times for IgG subclass secondaries were used. (E) Western blot showing IgM levels in AAB preparations. (F) Raw fluorescence intensity values for anti-human-Alexa488 detecting IgLON5 AAB binding to neuronal cultures. Note, values differ between curves because binding curves were determined at different experimental days using different microscope. (G) Partial co-localization of α -IgLON5#1 with pre-synaptic (Synapsin-1) and post-synaptic (PSD-95) markers. Scale bars as indicated. Data points are individual images analyzed. N = 3 independent experiments with 3-5 technical repeats each. Student t-test, ns = not significant. (H) Binding of α -IgLON5#1 to human iPSC-derived neurons. (I) Binding affinity of α -IgLON5#1 to HEK cells recombinantly expressing full-length human IgLON5 (IgLON5+; EC₅₀ = 8 μ g/ml; K_D = 55.6 nM) and of controls. Fluorescence quantified by FACS of cells after treatment. Data shown as mean \pm SEM, N = 3 independent experiments. (J) shRNA-mediated IgLON5 knockdown in Neuro2a cells. Reduced α -IgLON5#1 immunofluorescence indicates knockdown in mIgLON5. Cell membranes were stained with CellBrite. (K) Binding of α -IgLON5#1 to different IgLON5 IgG domains. Domain structure of human IgLON5 protein (336 amino acids) is shown, consisting of a secretion signal peptide (SP), three extracellular Ig domains (Ig1/2/3), and a C-terminal GPI-anchor. Example images show binding of α -IgLON5#1/ anti-human IgG-Alexa488 to HEK cells recombinantly expressing full-length IgLON5 protein or (combinations of) individual Ig domains. Scale bars as indicated.

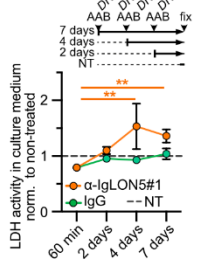
A Tau aggregation in FTD-Tau expressing neurons (1 µg/ml of AABs, 7 days)



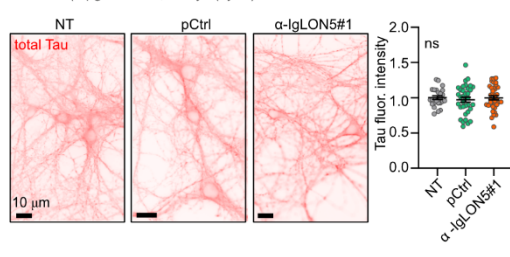
B Example images: Total Tau and phospho-Tau in neuronal cell bodies treated with different concentrations of α-IgLON5#1 for 2 days



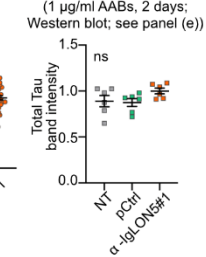
C α-IgLON5#1 neurotoxicity (1 µg/ml AABs)



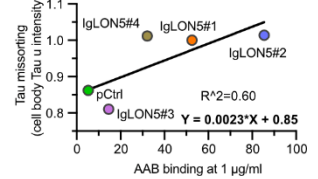
D Total Tau levels in neurons treated (1 µg/ml AABs, 2 days) (by IF)



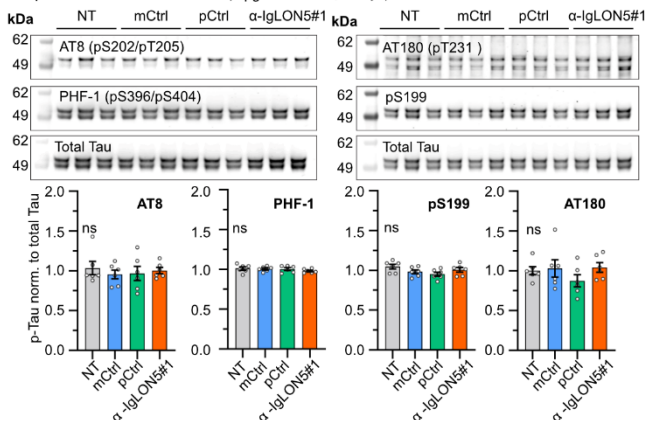
E Tau levels in neuronal lysates (1 µg/ml AABs, 2 days; Western blot, see panel (e))



F Correlation: α-IgLON5 AAB binding vs. Tau missing



G p-Tau in cultured neurons (1 µg/ml of AABs, 2 days)



H p-Tau in cultured neurons (1-20 µg/ml α-IgLON5#1, 2 days)

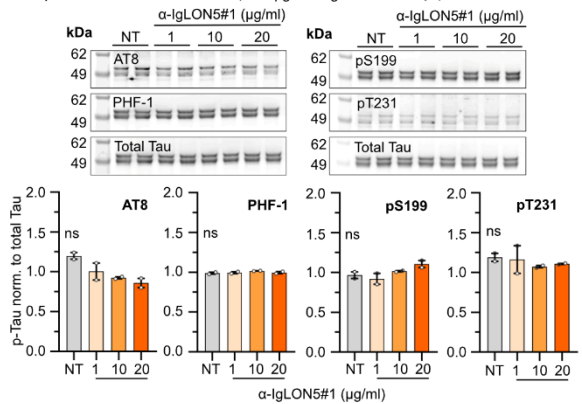


Fig. S2. Tau in AAB-treated neurons.

(A) Representative images of neurons expressing single FTD-mutant Tau variants, eGFP-TauDK280 and eGFP-TauP301L, or eGFP alone treated with α -IgLON5#1 or not. In no condition, Tau aggregates could be found. (B) Representative images of neuronal cell bodies stained for MAP2, total Tau and phospho-Tau after treatment with 0, 1, or 10 μ g/ml α -IgLON5#1 for 2 days. (C) Time-dependent toxicity (LDH assay) in neurons treated with 1 μ g/ml α -IgLON5#1 for 60 min, 2 days, 4 days, or 7 days. N = 3 experiments with 3-4 replicates. Two-way ANOVA with Sidak post-test. (D,E) Total Tau levels in neurons upon treatment with α -IgLON5#1 or pCtrl (1 μ g/ml) for 2 days, measured by immunofluorescence (D) and Western blot (E). N = 3 experiments with 3 replicates. One-way ANOVA with Tukey post-test. (F) Correlation (linear regression for R² and p-values) between α -IgLON5 AAB binding at 1 μ g/ml and missorted Tau in cell bodies after treatment with 1 μ g/ml AABs for 2 days. Data points represent the different patient-derived α -IgLON5 AABs. (G) Western blots for phospho-Tau (p-Tau pS202/pT205 (AT8), pS396/pS404, pS199, and pT231 (AT180)) and total Tau levels in lysates from neurons treated with 1 μ g/ml α -IgLON5#1 or control antibodies for 2 days. mCtrl is a non-reactive human monoclonal control antibody (mGO). N = 2 experiments with 3 technical replicates per condition. One-way ANOVA with Tukey post-test. (H) Western blots for phospho-Tau (p-Tau pS202/pT205 (AT8), pS396/pS404, pS199, and pT231 (AT180)) and total Tau levels in lysates from neurons treated with 1, 10, or 20 μ g/ml α -IgLON5#1 or control antibodies for 2 days. N = 2 independent experiment with 3 replicates per condition. One-way ANOVA with Tukey post-test.

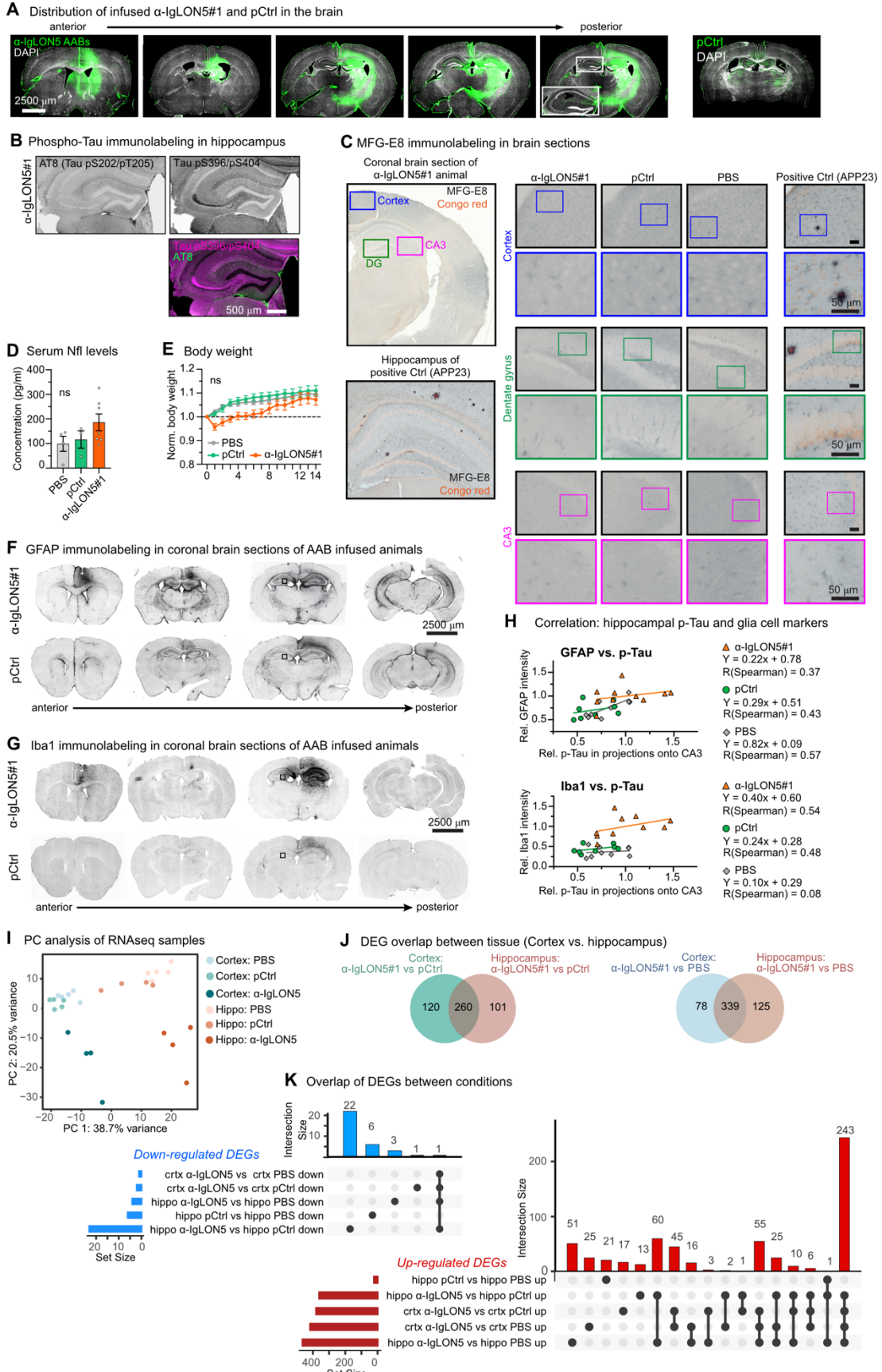
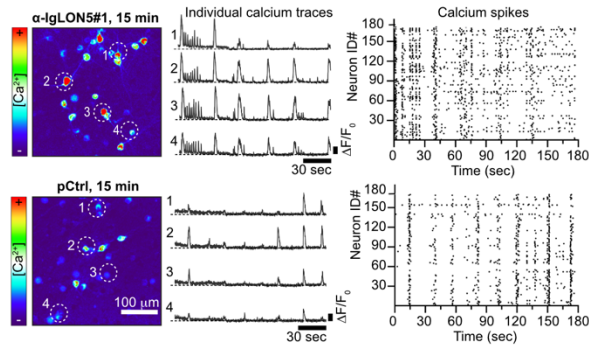


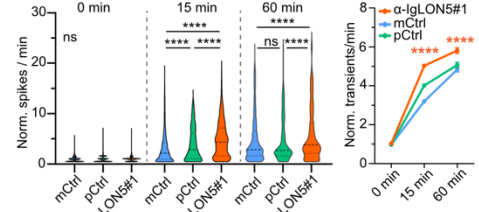
Fig. S3. Tau, toxicity, and neuroinflammation in mice infused with AABs.

(A) Representative coronal mouse brain sections (anterior to posterior) showing human antibody distribution (anti-human IgG) upon 14 days of unilateral cerebroventricular α -IgLON5#1 infusion. pCtrl-infused brain section is shown for comparison. (B) Representative images showing the hippocampal formation of an α -IgLON5#1 animal immunolabeled for Tau pS202/pT205 (AT8) and Tau pS396/pS404. (C) Representative images of cortex, DG and CA3 in α -IgLON5#1 and control brain sections immunolabeled for MFGE-8 and co-stained with Congo red. A brain section of an amyloid-beta expressing APP23 animal is shown as positive control. (D) Serum levels of neurofilament light chain (Nfl; by ELISA) are non-significantly elevated in α -IgLON5#1 versus pCtrl and PBS animals. Data shown as mean \pm SEM, data points represent individual animals. N = 3 - 7 animals with 3 technical replicates measured per animal. One-way ANOVA with Tukey post-test. (E) Body weight relative to Day 0 measured during 14 days of AAB infusion. Data shown as mean \pm SEM, N = 8 - 10 animals per group. Student t-test comparing α -IgLON5#1 versus pCtrl or PBS at each time point. (F) Representative series of coronal brain sections (anterior to posterior) from α -IgLON5#1 and pCtrl infused animals, immunolabeled for GFAP in astrocytes. Scale bar as indicated. (G) Iba1 (microglia) immunolabeling in the same brain sections shown in (F). Scale bar as indicated. (H) Correlation (linear regression) between hippocampal GFAP (top) or Iba1 (bottom) fluorescence intensities and Tau pS396/pS404 in projections onto CA3 for α -IgLON5#1, pCtrl, and PBS animals. Data points represent mean of individual animals. (I) Principal component analysis (PCA) of RNA-seq data obtained from contralateral cortical and hippocampal tissues of mice infused with PBS (N = 5), pCtrl (N = 4 - 5), or α -IgLON5#1 (N = 4) shows a clear separation of α -IgLON5#1 from control cortices and hippocampi. (J) Venn diagrams showing the number of unique and shared differentially expressed genes (DEGs) between cortex and hippocampus for α -IgLON5#1 vs. pCtrl (left), and α -IgLON5#1 vs. PBS (right). (K) UpSet plots of intersection analysis of down- (blue) and up- (red) regulated DEGs across experimental groups. The largest overlap (n = 243 DEGs) is found in upregulated DEGs between α -IgLON5#1 cortex and hippocampus vs. respective brain regions in PBS and pCtrl, indicating consistent upregulation of transcripts in hippocampus and cortex of α -IgLON5#1-treated animals. All scale bars as indicated.

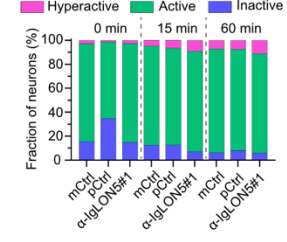
A Ca²⁺ imaging in neurons treated with 0.1 μg/ml AABs



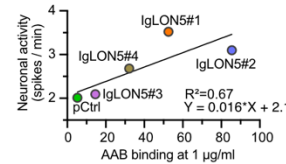
B Ca²⁺ spike frequency (0.1 μg/ml AABs)



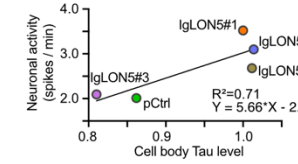
C Neuronal activity categories



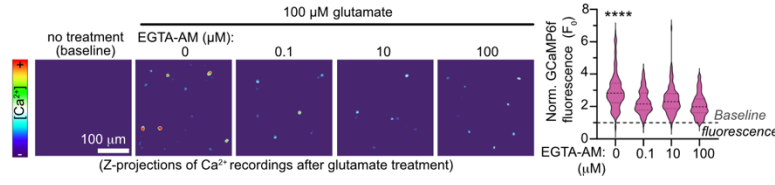
D Correlation: Activity vs. AAB binding



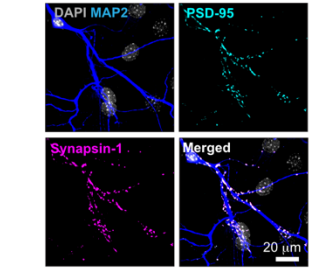
E Correlation: Activity vs. Tau missorting



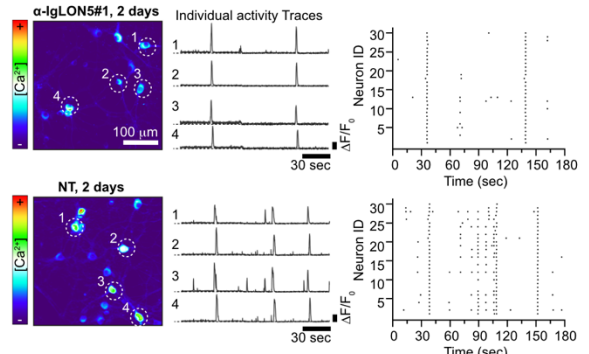
F Effects of Ca²⁺ chelation on GCaMP6f fluorescence after glutamate (100 μM) treatment



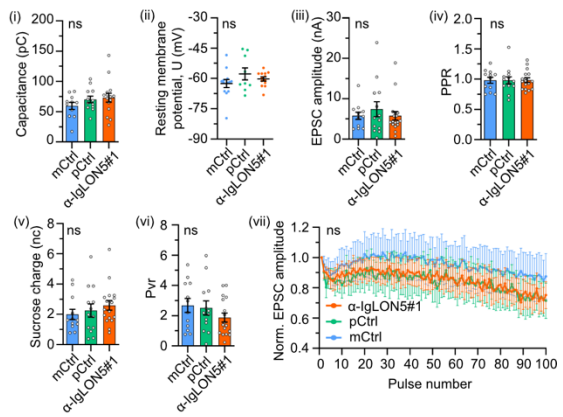
H Representative image of hippocampal neuron autaptic culture



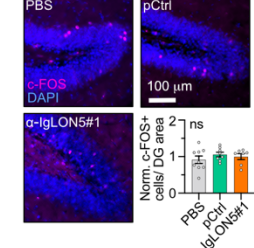
G Ca²⁺ imaging of neurons (1 μg/ml AABs, 2 days)



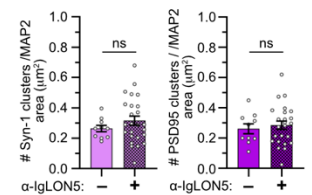
I Electrophysiology in autaptic cultures (0.1 μg/ml AABs, 3 days)



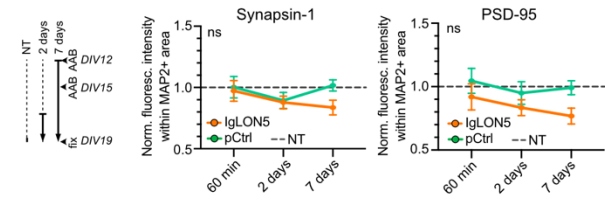
J c-FOS in dentate gyrus (DG)



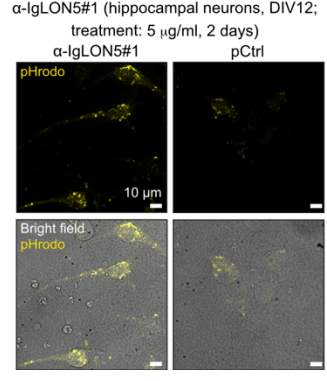
K Synapse density (1 μg/ml AAB, 60 min)



L Synapse density time course



M Internalization of pHrodo™-labeled α-IgLON5#1 (hippocampal neurons, DIV12; treatment: 5 μg/ml, 2 days)



N Neuronal α-IgLON5 distribution (60 min)

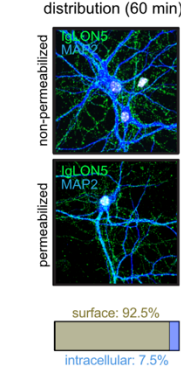


Fig. S4. Supplementary Figure S4. Activity of AAB-treated neurons.

(A) Representative images showing Ca^{2+} levels (time-integrated; pseudo-colored) derived from time-lapse recordings of GCamp6f expressing neurons treated with 0.1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5\#1}$ or pCtrl for 15 min. White outlined ROIs indicate the position of the neurons for which GCamp6f intensity traces ($\Delta\text{F}/\text{F}_0$ vs time) are shown. Raster plots show the occurrence of individual Ca^{2+} spikes in cell bodies of all cells in one field of view ($N \sim 180$ cells, y-axis). Scale bars as indicated.

(B) Spike rates in neurons treated with 0.1 $\mu\text{g}/\text{ml}$ of $\alpha\text{-IgLON5\#1}$, pCtrl, or mCtrl (non-reactive monoclonal human antibody mGO (37)) for 15 min and 60 min. Ca^{2+} spikes were recorded before (0 min) and after 15 min and 60 min of AAB treatment in the same cultures. Data shown as mean \pm SEM, $N = 3$ independent experiments, 71-994 neurons per group. One-way ANOVA with Tukey post-test.

(C) Percentage of inactive (blue), active (green), and hyperactive (pink) neurons in cultures treated with 0.1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5\#1}$, pCtrl and mCtrl for 0 min, 15 min or 60 min. Hyperactive criteria: # of spikes/min $>$ mean $+2$ *SD of pCtrl at each recording time point. Active: $0 <$ # of spikes/min $<$ mean $+2$ *SD of pCtrl at each recording time point. Silent: no spikes.

(D) Correlation (linear regression) between neuronal activity and AAB binding after treatment with 1 $\mu\text{g}/\text{ml}$ AABs for 60 min. Data points represent $\alpha\text{-IgLON5}$ AABs obtained from different patients.

(E) Correlation (linear regression) between neuronal activity after treatment with 1 $\mu\text{g}/\text{ml}$ AABs for 60 min and cell body Tau levels after treatment with 1 $\mu\text{g}/\text{ml}$ AABs for 2 days. Data points represent $\alpha\text{-IgLON5}$ AABs obtained from different patients.

(F) Representative images showing Ca^{2+} levels (time-integrated; pseudo-colored) derived from time-lapse recordings in GCamp6f -expressing hippocampal neurons, pre-treated with increasing concentrations of EGTA-AM (0.1-100 μM) for 20 min followed by glutamate (100 μM) stimulation for 20 min. Violin plots show GCamp6f fluorescence intensities in neurons. $N = 90$ -124 neurons per group were analyzed. One-way ANOVA with Tukey's post-test. Scale bar as indicated.

(G) Representative images showing Ca^{2+} levels (time-integrated; pseudo-colored) derived from time-lapse recordings, individual activity traces, and raster plots for detected Ca^{2+} spikes in neurons treated with 1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5\#1}$ for 2 days or not treated (NT). Scale bars as indicated.

(H) Representative image of autaptic cultures immunolabeled for DAPI, MAP2, PSD95 and Synapsin-1. Scale bars as indicated.

(I) Electrophysiological recordings in autaptic cultures treated with 0.1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5}$ AABs, pCtrl and mCtrl for 3 days. mCtrl is a non-reactive human monoclonal control antibody. Measured parameters are (i) membrane capacitance (pC), (ii) resting membrane potential, (iii) excitatory postsynaptic current (EPSC) amplitude, (iv) EPSC paired-pulse ratio (PPR), (v) readily releasable pool size (sucrose charge), (vi) synaptic vesicle release probability (Pvr), and (vii) EPSC release during 10 Hz stimulation. $N = 3$ independent autaptic cultures. Data shown as mean \pm SEM. One-way ANOVA with Tukey post-test.

(J) Immunolabeling of c-FOS in the DG of $\alpha\text{-IgLON5\#1}$ and control animals. Data shown as mean \pm SEM, $N = 7$ -9 animals, 3-5 brain sections per animal. One-way ANOVA with Tukey post-test. Scale bar = 100 μm .

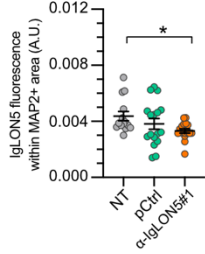
(K) Pre-(Synapsin-1) and post- (PSD95) synaptic cluster densities (# punctae/MAP+ area) in neurons treated with 1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5\#1}$ for 60 min, or not. $N = 3$ experiments with 3-5 images analyzed per condition and experiment. Data shown as mean \pm SEM, Student t-test.

(L) Analysis of synaptic marker (Synapsin-1 and PSD-95) densities upon incubation of neurons with 1 $\mu\text{g}/\text{ml}$ $\alpha\text{-IgLON5\#1}$ for 60 min, 2 days, or 7 days. Data shown as mean \pm SEM, $N = 3$ experiments with 3 replicates per experiment and 3-5 images per replicate. Dashed lines indicate corresponding synaptic marker levels of untreated neurons at DIV19.

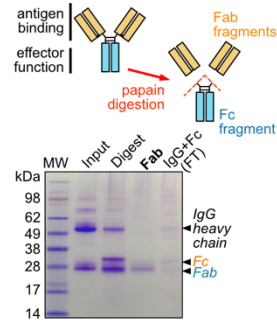
(M) Representative images showing internalization and lysosomal acidification (= fluorescence) of pHrodoTM-labelled $\alpha\text{-IgLON5\#1}$ or pCtrl in primary

hippocampal mouse neurons after 2 days treatment. Scale bars indicated. (N) Representative images for immunolabeling of cell surface α -IgLON5#1 in unpermeabilized and total α -IgLON5#1 in permeabilized (TX-100) neurons. Percentage of surface vs. internalized α -IgLON5#1 clusters after 60 min of treatment was calculated from 3 images each. Note, image of unpermeabilized neurons is the same as in Fig. 1D.

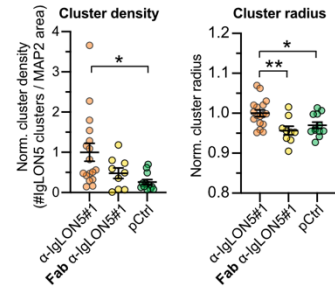
A Total cell surface α -IgLON5#1 fluorescence after 60 min AAB treatment



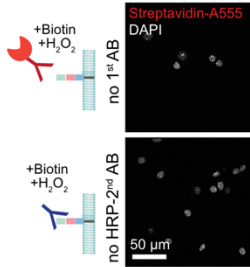
B Fab fragments of α -IgLON5#1



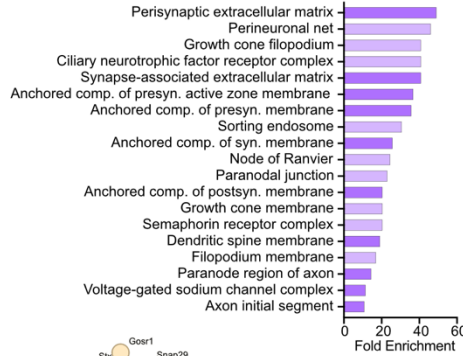
C α -IgLON5#1 Fab fragment effects on IgLON5 clusters



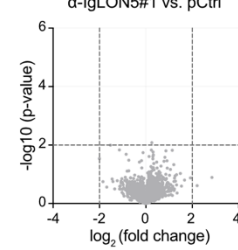
D Additional controls for cluster proteomics



F GO Cellular Components



G Input (lysate) proteome: α -IgLON5#1 vs. pCtrl



E String network of IgLON5 cluster components

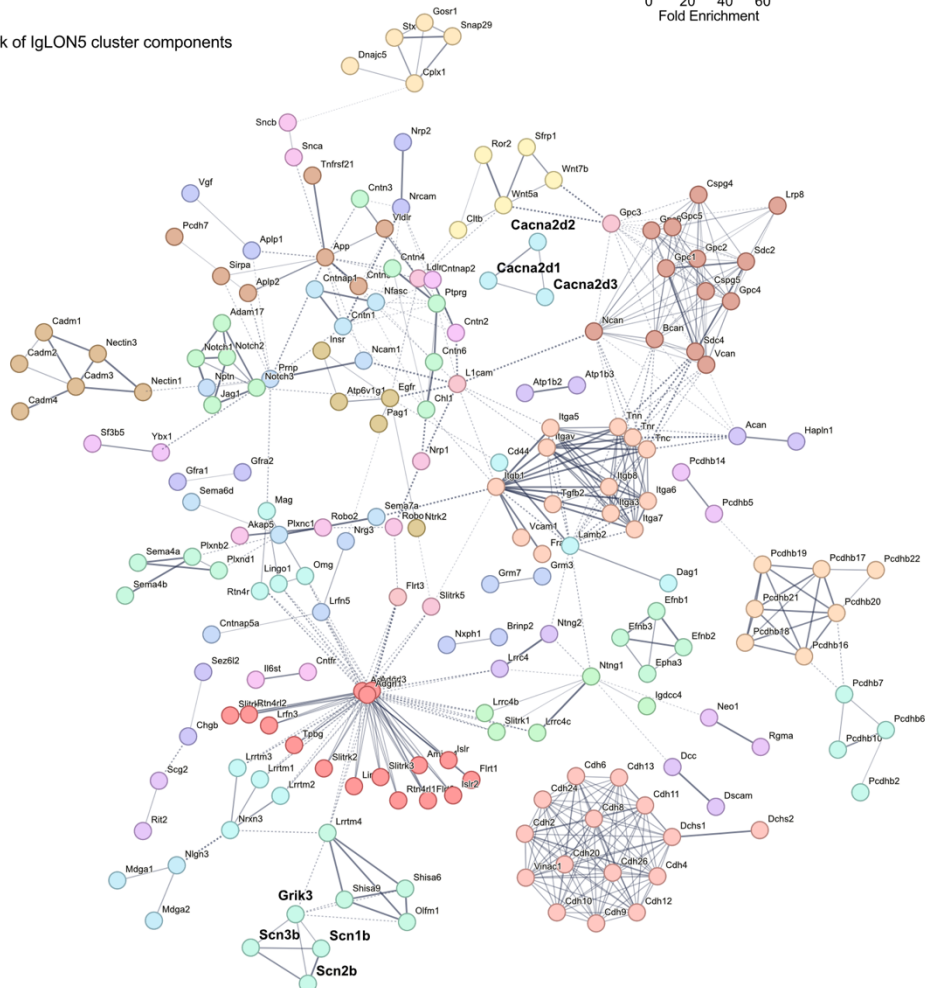


Fig. S5. α -IgLON5 AAB Fab fragments and IgLON5 surface cluster analysis.

(A) Total fluorescence intensity of α -IgLON5#1 on the neuronal cell surface in fixed neurons with and without treatment of IgLON5 prior to fixation. Data shown as mean \pm SEM, data points represent images analyzed. N = 3 experimental and 3-5 technical replicates. One-Way ANOVA with Tukey post-test. (B) Principle and representative reducing SDS-PAGE analysis of Fab fragment generation by papain digestion. Lane-1: input = undigested IgG, Lane-2: papain digested IgG-containing reduced Fc and Fab fragments, Lane-3: purified and reduced IgG Fab fragments (~25 kDa), and Lane-4: flow-through (FT) containing undigested IgG, Fc and Fab fragments. (C) Normalized IgLON5 surface cluster density (# IgLON5 clusters/ MAP2+ area) and radius in neurons treated with 1 μ g/ml undigested pCtrl or α -IgLON5#1 (~150 kDa, containing 2 Fab fragments per IgG), or with 1.5 μ g/ml isolated, non-reduced Fab α -IgLON5#1 (~50 kDa per Fab fragment) for 60 min. N = 2 independent experiments with 3 replicates each and 3 images per replicate. Data shown as mean \pm SEM. One-way ANOVA with Tukey's post-test. (D) Representative images showing no biotinylation in control conditions of IgLON5 surface cluster proximity labeling lacking either the α -IgLON5#1 primary or the HRP-coupled anti-human secondary. (E) Visualization of protein-protein interaction network (STRING) of proteins identified in the IgLON5 cluster proteome. (F) Gene Ontology (GO) enrichment analysis of cellular components associated with proteins significantly enriched in α -IgLON5 AAB-induced surface clusters. (G) Volcano plot for input (total lysate) samples comparing α -IgLON5#1 vs. pCtrl.

Table S1. Antibody list.

IF: Immunofluorescence IHC: Immunohistochemistry WB: Westernblot						
Primary antibody	Species	Catalog number	company	Application and dilution		
				IF	IHC	WB
MAP2	chicken	ab92434	Abcam	1:1000		
Synapsin-1	guinea pig	106004	Synaptic Systems	1:500		
PSD-95	mouse	MAB159	Millipore	1:1000		
GFAP	mouse	MAB3402	Millipore		1:500	
Iba1	rabbit	019-19741	WAKO		1:500	
CD68	rat	MCA1957T	BioRad		1:500	
Complemet3 (C3)	rat	HM1045	HycultBiotech		1:100	
C1q	rabbit	ab182451	Abcam		1:1000	
CD8	rat	MA1-145	Invitrogen		1:100	
Caspase-3	rabbit	559565	BD Pharmingen		1:500	
MFG-E8	goat	AF2805	R&D Systems		1:1000	
c-FOS	rat	226 017	Synaptic systems	1:1000	1:500	
Biotin	rabbit	5597S	CellSignaling			1:1000
GAPDH	mouse	CB1001	Millipore			1:1000
Tau-5	mouse	MA5-12808	Invitrogen	1:1000		1:5000
Tau (DAKO)	rabbit	10024	DAKO			1:5000
p-Tau (Ser396)*	rabbit	ab109390	Abcam		1:1000	1:5000
pTau (Ser404)*	rabbit	ab92676	Abcam		1:500	1:2000
p-Tau (Ser202)** +	rabbit	39357S	Cell Signaling Technology		1:500	1:1000
p-Tau (Thr205)**	rabbit	44-738G	Invitrogen		1:500	1:1000
p-Tau (Thr231) (AT180)	mouse	MN1040	Invitrogen			1:500
p-Tau (S202/S205) (AT8)	mouse	MN1020	Invitrogen			1:1000
p-Tau (Ser199)	rabbit	ab4749	Abcam			1:1000
<i>*Mixed to mimick PHF-1 epitope; **Mixed to mimick AT8 epitope; + This antibody didn't work for IHC</i>						
Secondary antibody	Catalog number	company	Application and dilution			
			IF	IHC	WB	
Goat anti-human 488	109-545-003	Jackson ImmunoResearch	1:500			
Goat anti-Mouse, Alexa Fluor 488	A-21121	Invitrogen	1:1000			

Goat anti-Rat, Alexa Fluor 488	A-1106	Invitrogen		
Goat anti-Guinea Pig, Alexa Fluor 488	A-11073	Invitrogen		
Goat anti-Rat, Alexa Fluor 555	A-21434	Invitrogen		
Goat anti-Chicken, Alexa Fluor Plus 555	A-32932	Invitrogen		
Goat Anti-Rabbit, Alexa Fluor 647	A-21245	Invitrogen	1:500	
Streptavidin, Alexa Fluor 555	S32355	Invitrogen	1:500	
Goat anti-Mouse, Alexa Fluor 790	A11375	Invitrogen		1:20 000
Goat anti-Rabbit, Alexa Fluor 790	A11367	Invitrogen		1:20 000
Goat anti-Mouse, DyLight 680	35519	Invitrogen		1:10 000
Goat anti-Rabbit, DyLight 680	35569	Invitrogen		1:10 000
Goat anti-human HRP	31410	Invitrogen		1:200
			1:1000 (Proximity biotinylation)	