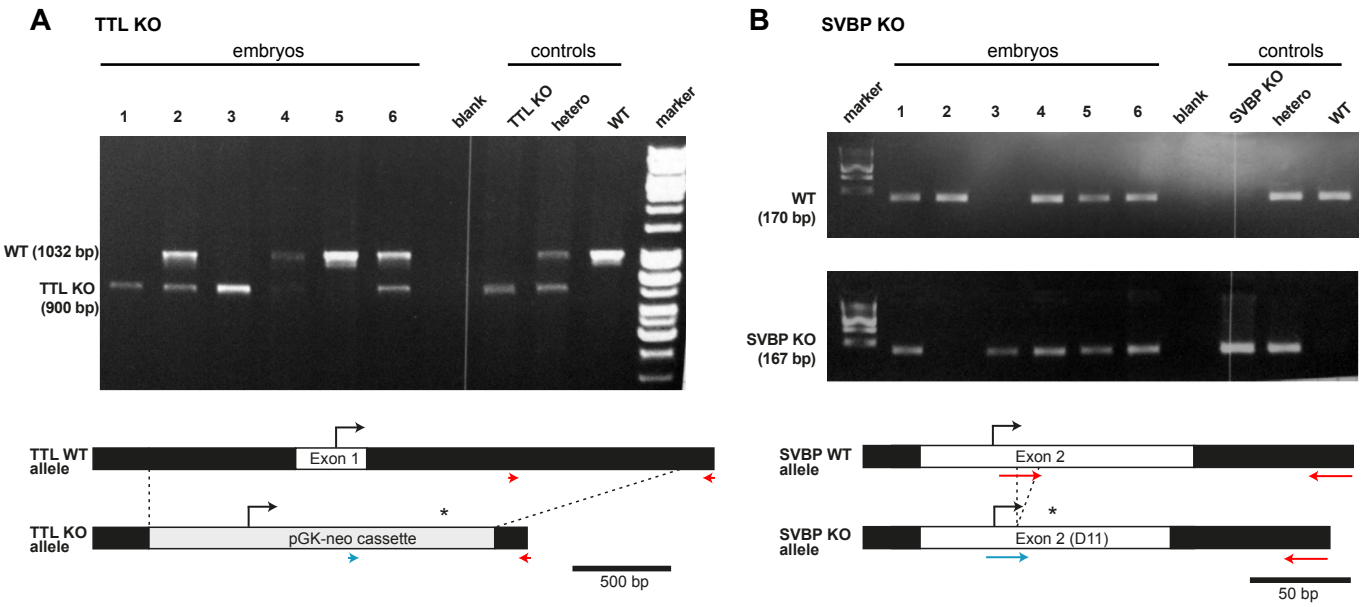


Fig. S1. TTL-KO and SVBP-KO genotyping and protein levels of AnkG and TRIM46. A) Exon 1 of TTL was replaced by a neomycin resistance cassette driven by phosphoglycerate kinase I promoter (pGK-neo) as described in Erck et al. (2005). B) A frameshift was introduced in exon 2 of SVBP, causing a premature stop codon (*), as described in Pagnamenta et al. (2019). For details on genotyping primers, see Material & Methods.

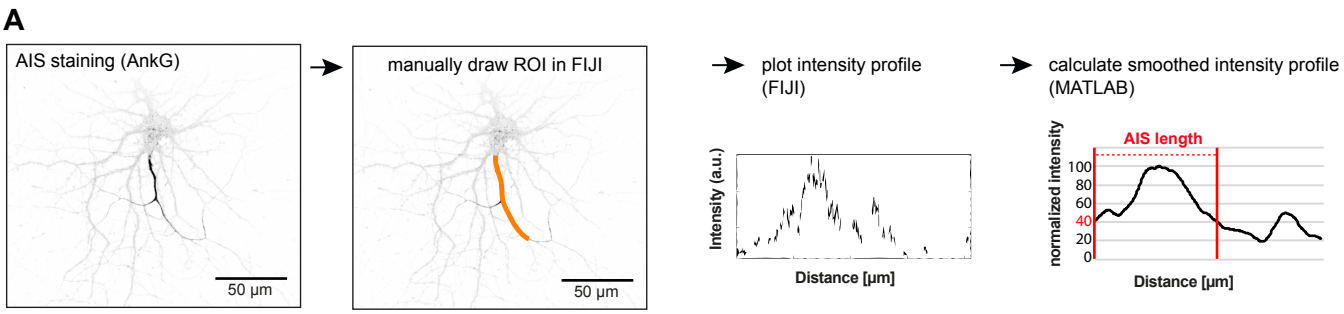


Fig. S2. Overview of the workflow for AIS length calculation.

A) Neurons were stained with an AIS marker (AnkG or TRIM46). A ROI was drawn manually from the cell body over the entire length of the marker region in FIJI. Raw intensity profiles from FIJI were smoothed in MATLAB using a 3-pixel sliding window and normalized to 100%. Start and end of the AIS were defined as the points where the normalized intensity crossed above or below 40% of the maximum intensity. Scale bar = 50 um.

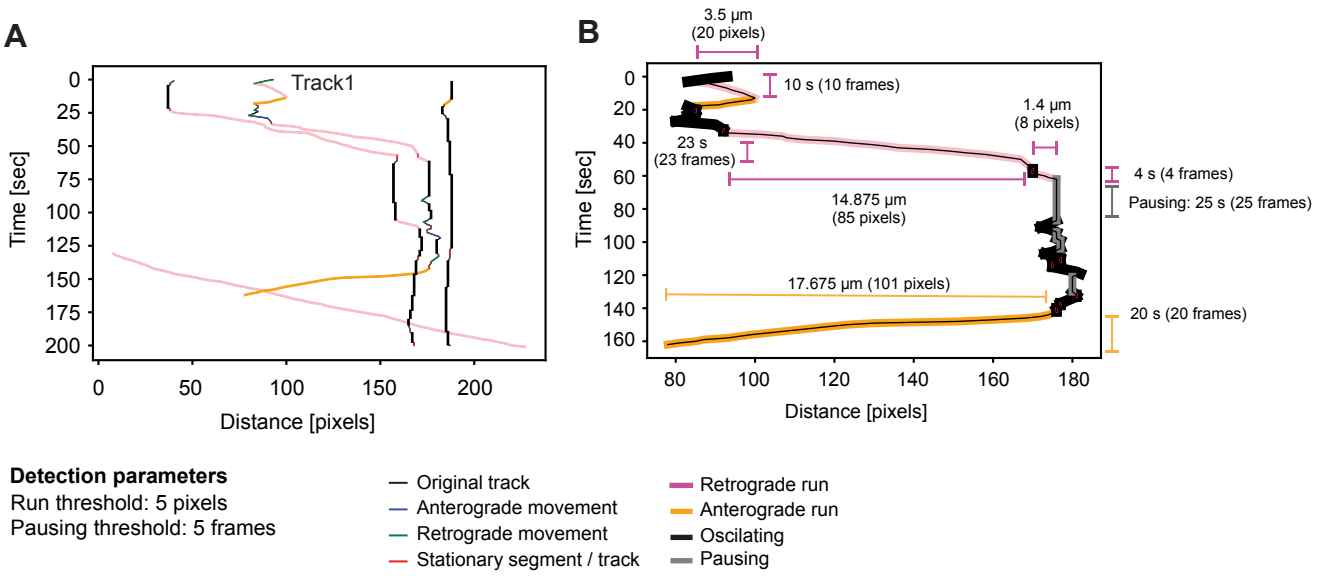


Fig. S3. Semi-automated analysis of lysotracker-positive vesicles. A) Example of kymograph reconstruction via the KYMOA program (https://github.com/HU-Berlin-Optobiology/AIS-project.git) showing Lysotracker-vesicle trajectories in the axonal compartment of a mouse hippocampal neuron. Each track represents the trajectory of one Lysotracker-positive vesicle. B) Annotation of track 1. In total, 3 processive retrograde runs, 2 processive anterograde runs, 3 pausing events and 6 oscillating movements were identified with the detection parameters indicated at the bottom of the figure.

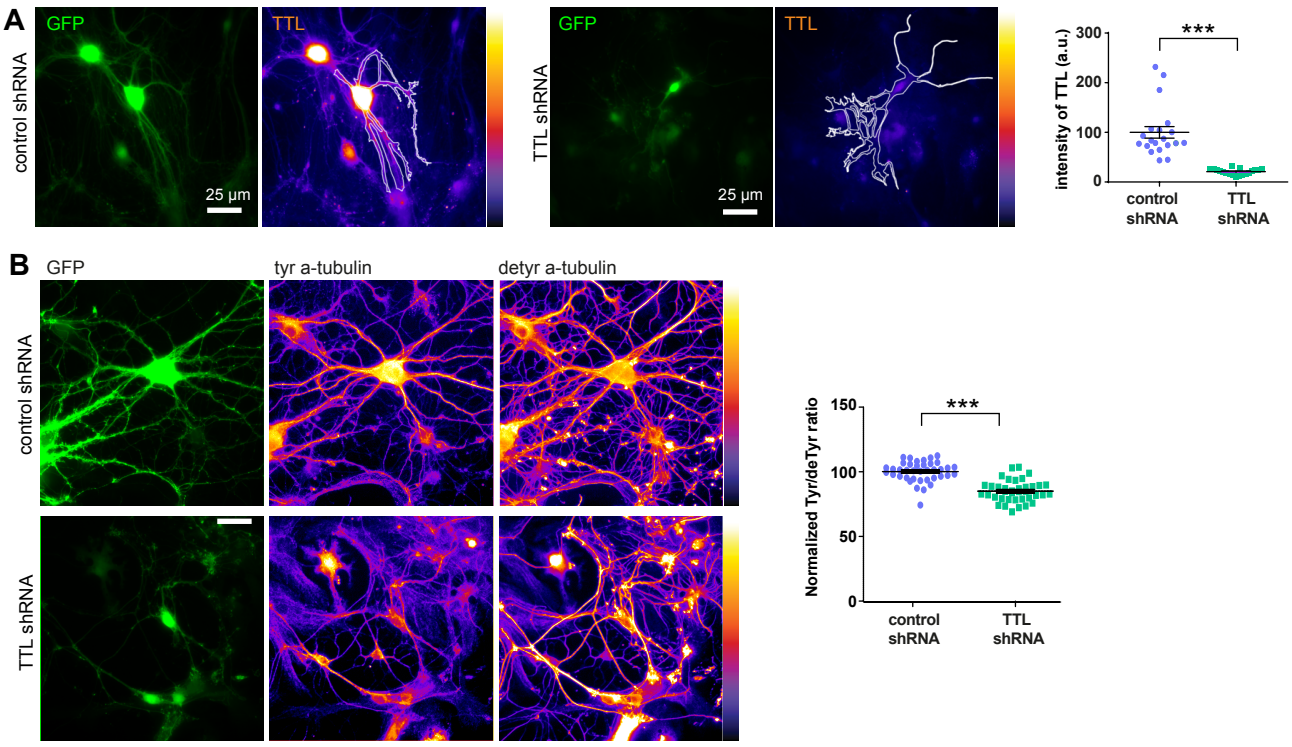


Fig. S4. Acute shRNA knockdown of TTL in mouse hippocampal neurons. A) Knockdown of TTL leads to reduced detected TTL levels in DIV 14 neurons. Cell outline is marked with white mask. **B)** Knockdown of TTL decreases the Tyr/deTyr ratio in DIV14 neurons. Scale bars = 25 µm. (A) n = 20, 19 cells from control (1 independent culture) and TTL shRNA (1 independent culture). (B) n = 39, 38 cells from control (2 inde-pendent cultures) and TTL shRNA (2 independent cultures). Error bars show mean ± SEM.

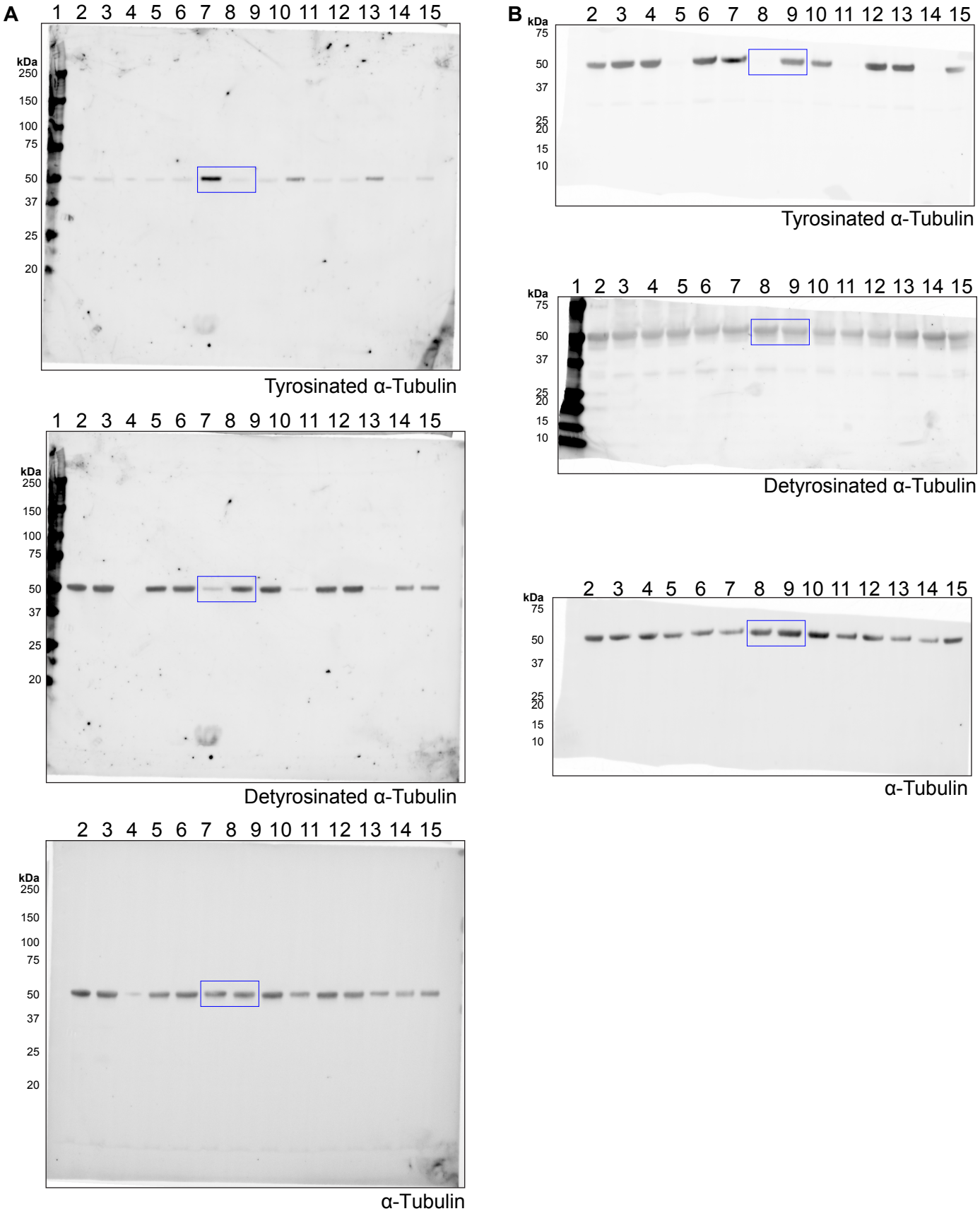


Fig. S5. Western blot Transparency corresponding to Fig. 1.

A) Whole membranes corresponding to Figure 1 B), blue boxes correspond to insets shown in Fig. S1 C), insets have been flipped horizontally and contrast was increased. 1 Protein Ladder, 2 WT, 3 SVBP het., 4 SVBP KO, 5 WT, 6 SVBP het., 7 SVBP KO, 8 WT, 9 SVBP het., 10 SVBP KO, 11 WT, 12 SVBP het., 13 SVBP KO, 14 WT, 15 WT+TTL. **B)** Whole membranes corresponding to Figure 1 C), blue boxes correspond to insets shown in supplemental Figure 1 C), insets have been flipped hori-zontally and contrast was increased. 1 Protein Ladder, 2 CI, 3 WT, 4 TTL het., 5 TTL KO, 6 WT, 7 TTL het., 8 TTL KO, 9 WT, 10 TTL het., 11 TTL KO, 12 WT, 13 TTL het., 14 TTL KO, 15 CI.

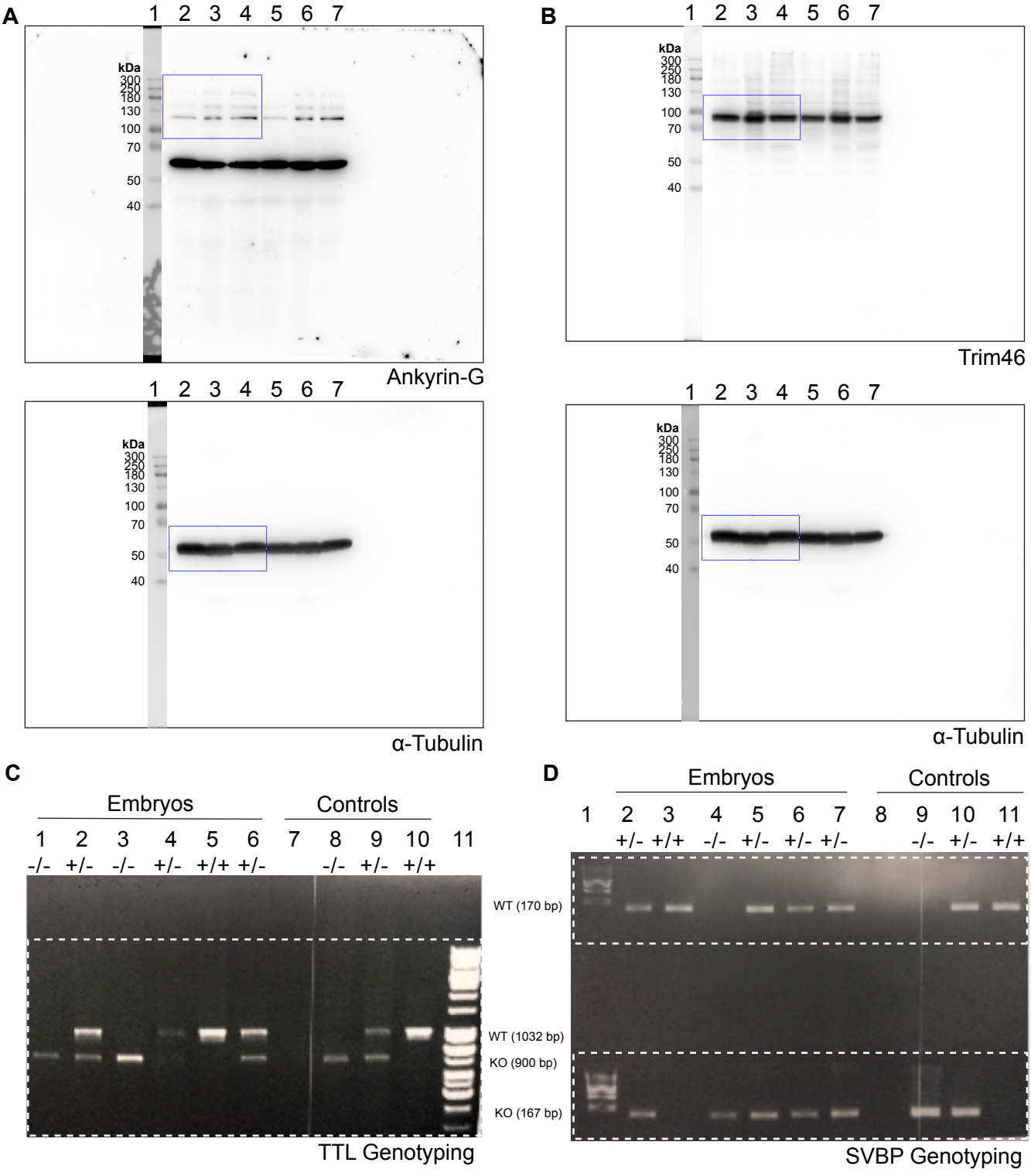
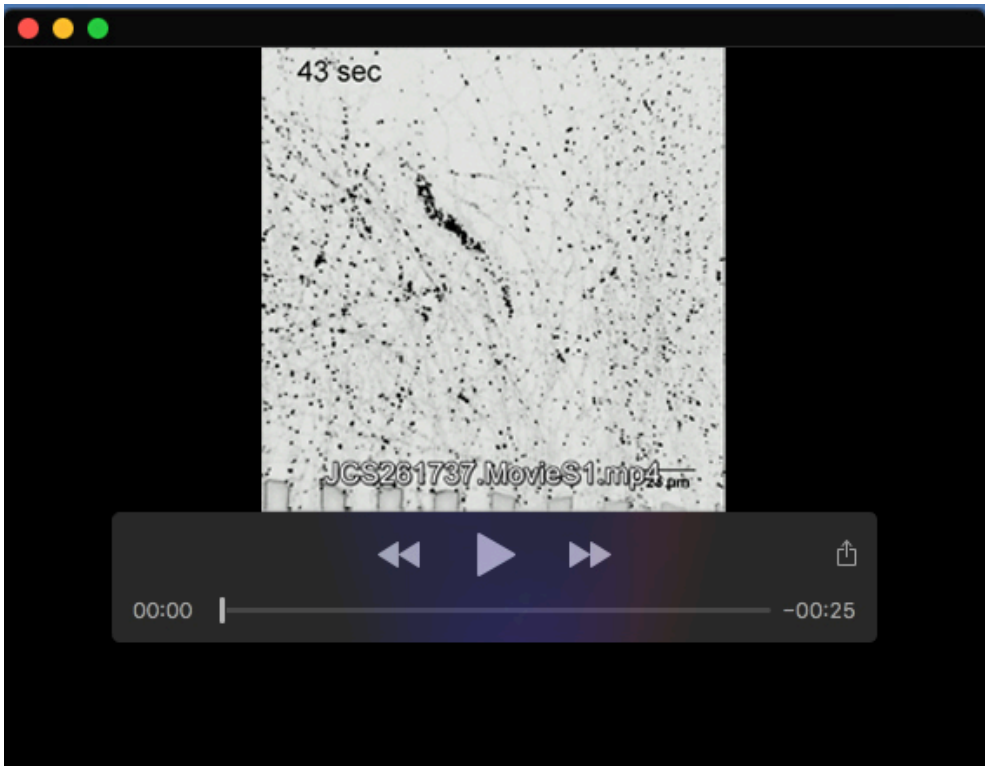
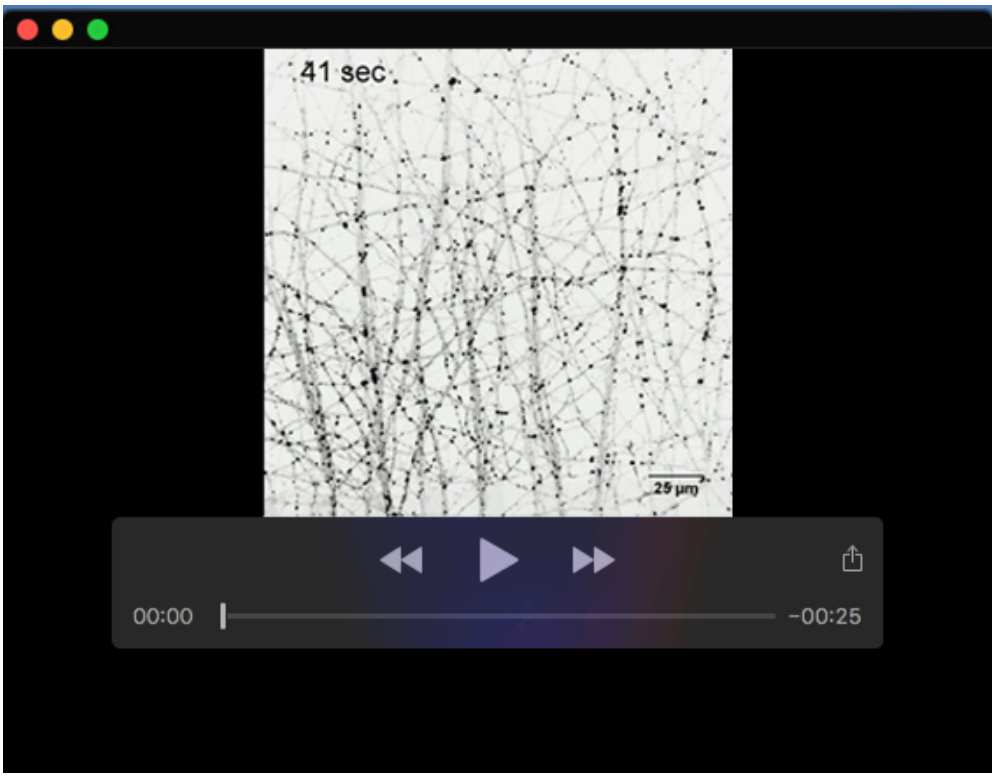


Fig. S6. Western blot and agarose gel transparency corresponding to Fig. 2 and Fig. S1
A) Whole membranes corresponding to Figure 2 A), blue boxes correspond to insets shown in Figure 2 A), insets have been flipped horizontally. 1 Spectra High-Range, 2 TTL KO, 3 WT, 4 SVBP KO, 5 TTL KO, 6 WT, 7 SVBP KO. **B)** Whole membranes corresponding to Figure 2 B), blue boxes correspond to insets shown in Figure 2 B), insets have been flipped horizontally. 1 Spectra High-Range, 2 TTL KO, 3 WT, 4 SVBP KO, 5 TTL KO, 6 WT, 7 SVBP KO. **C)** Whole agarose gel corresponding to Supplemental Figure 1 A), dashed line box corresponds to inset shown in Supplemental Figure 1 A). 1 Embryo #1: TTL KO, 2 Embyro #2: TTL het., 3 Embryo #3: TTL KO, 4 Embyro #4: TTL het., 5 Embyro #5: WT, 6 Embryo #6: TTL het., 7 blank control, 8 TTL KO control, 9 TTL het. control, 10 WT control, 11 DNA marker. **D)** Whole aga-rose gel corresponding to Supplemental Figure 1 B), dashed line boxes correspond to insets shown in Supplemental Figure 1 B). 1 DNA marker, 2 Embyro #1: SVBP het., 3 Embryo #2: WT, 4 Embyro #3: SVBP KO, 5 Embyro #4: SVBP het., 6 Embryo #5: SVBP het., 7 Embryo #6: SVBP het., 8 blank control, 9 SVBP KO control, 10 SVBP het. control, 11 WT control.

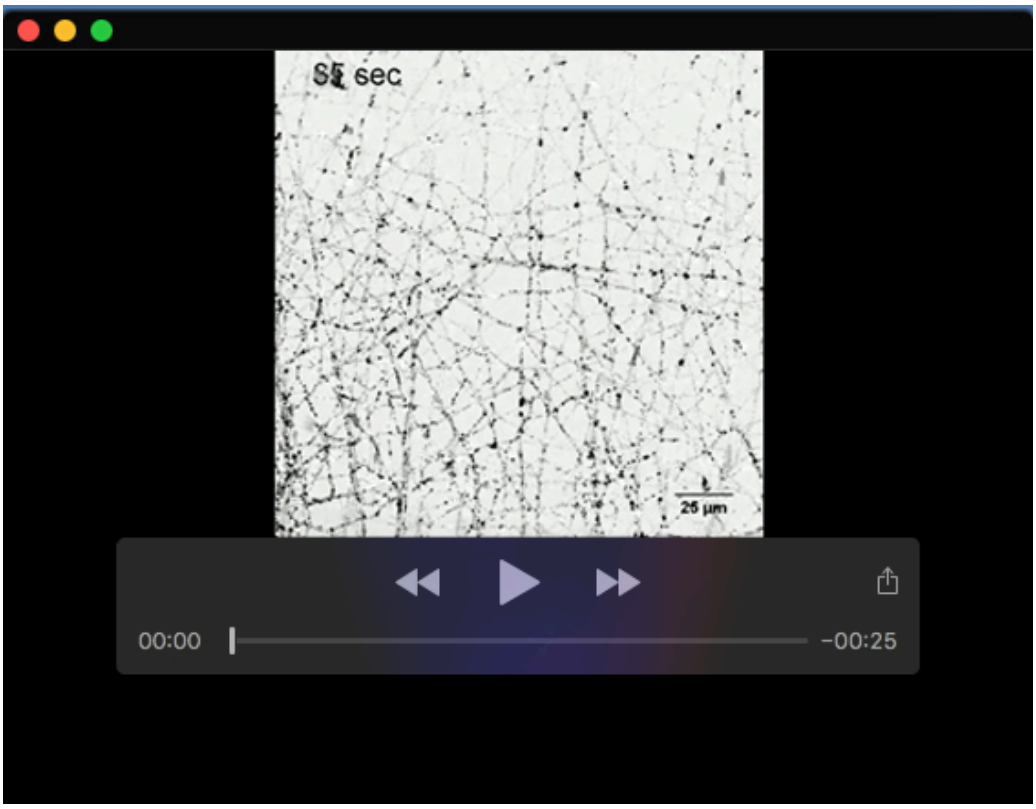


Movie 1. Corresponding to Fig. 4B. Movement of lysotracker-positive vesicles in the axonal compartment of SVBP KO neurons grown in 2-compartment MFCs imaged at 1 frame per second. Scale bar = 25 µm.



Movie 2. Corresponding to Fig. 4C. Movement of lysotracker-positive vesicles in the axonal compartment of WT neurons grown in 2-compartment MFCs imaged at 1 frame per second. Scale bar = 25 µm.

Journal of Cell Science • Supplementary information



Movie 3. Corresponding to Fig. 4D. Movement of lysotracker-positive vesicles in the axonal compartment of TTL KO neurons grown in 2-compartment MFCs imaged at 1 frame per second. Scale bar = 25 µm.

Table S1. Crucial reagents and resources Primary antibodies

Confocal and STED imaging

Target	Host	Supplier	Product number	Dilution
AnkyrinG	Mouse	Millipore	MABN466	1:500
TRIM46	Rabbit	Synaptic Systems	377 003	1:500
Beta-4-spectrin	Mouse	Neuromab	75-377	1:500
MAP2	Mouse	Sigma	M4403	1:500
MAP2	Rabbit	Abcam	ab32454	1:500
Detyr-α-tubulin	Rabbit	Abcam	ab48389	1:500
Tubulin Tyrosine Ligase	Mouse	Proteintech	66076-1-Ig	1:500

Western Blot – Fig. 1

α-tubulin (α3A1)	Mouse	*Self-made (described in Aillaud et al., 2017)*	n.a.	1:10000
Tyr-α-tubulin (YL1/2)	Rat		n.a.	1:10000
Detyr-α-tubulin	Rabbit		n.a.	1:10000

Western Blot – Fig. 2

Ankyrin-G	Mouse	EMD Millipore	MABN466	1:500
TRIM46	Rabbit	Proteintech	30298-1-AP	1:2000
α-tubulin	Mouse	Sigma	T5168	1:2000

Secondary antibodies

Confocal Imaging

Target	Host	Conjugation	Supplier	Product number	Dilution
Mouse	Goat	Alexa Fluor 568	Thermo Fisher	A-11031	1:500
Rabbit	Goat	Alexa Fluor 488	Thermo Fisher	A-21245	1:500
Mouse	Goat	Alexa Fluor 594	Thermo Fisher	A-11005	1:500

STED Imaging

Mouse	Goat	Alexa Fluor 594	Thermo Fisher	A-11005	1:500
Mouse	Goat	Abberior Star Orange	Fisher Scientific	NC1933863	1:500

Western Blot

Mouse	Goat	HRP	Dianova	115-035-146	1:10 000
Rabbit	Goat	HRP	Dianova	111-035-144	1:10 000

Other reagents

Name	Supplier	Product number	Dilution
DAPI	Sigma	D9542	1:1000
Lysotracker Green DND-26	Thermo Fisher	L7526	1:10.000
Lysotracker Red DND-99	Thermo Fisher	L7528	1:20.000
Magic Red Cathepsin-B Assay Kit	Biomol	ICT937	1:250
Phalloidin-Atto647N	Sigma	65906	1:50

AAVs

Name	Supplier	Viral titer	Dilution
AAV9 sh_5027 (control shRNA) produced from pAAV9-U6-shRNA_5027-EF1α-eGFP shRNA sequence: GGAATCTCATTCGATGCATACCTC GAGGTATGCATCGAATGAGATTCC	Servier	7.0^12 viral genomes/ml	1:8000
AAV9 sh_3616 (mouse TTL shRNA) produced from pAAV9-U6-shRNA_3616-EF1α-eGFP shRNA sequence: AAGTGCACGTGATCCAGAAATCTC GAGATTTCTGGATCACGTGCACTT	Servier	1.5^13 viral genomes/ml	1:8000

References

Aillaud et al. Vasohibins/SVBP are tubulin carboxypeptidases (TCPs) that regulate neuron differentiation. *Science*. 2017 Dec 15; **358** (6369):1448-1453.