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Supplemental Information

Wnt Signaling Directs Neuronal Polarity and Axonal Growth

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Figure S1

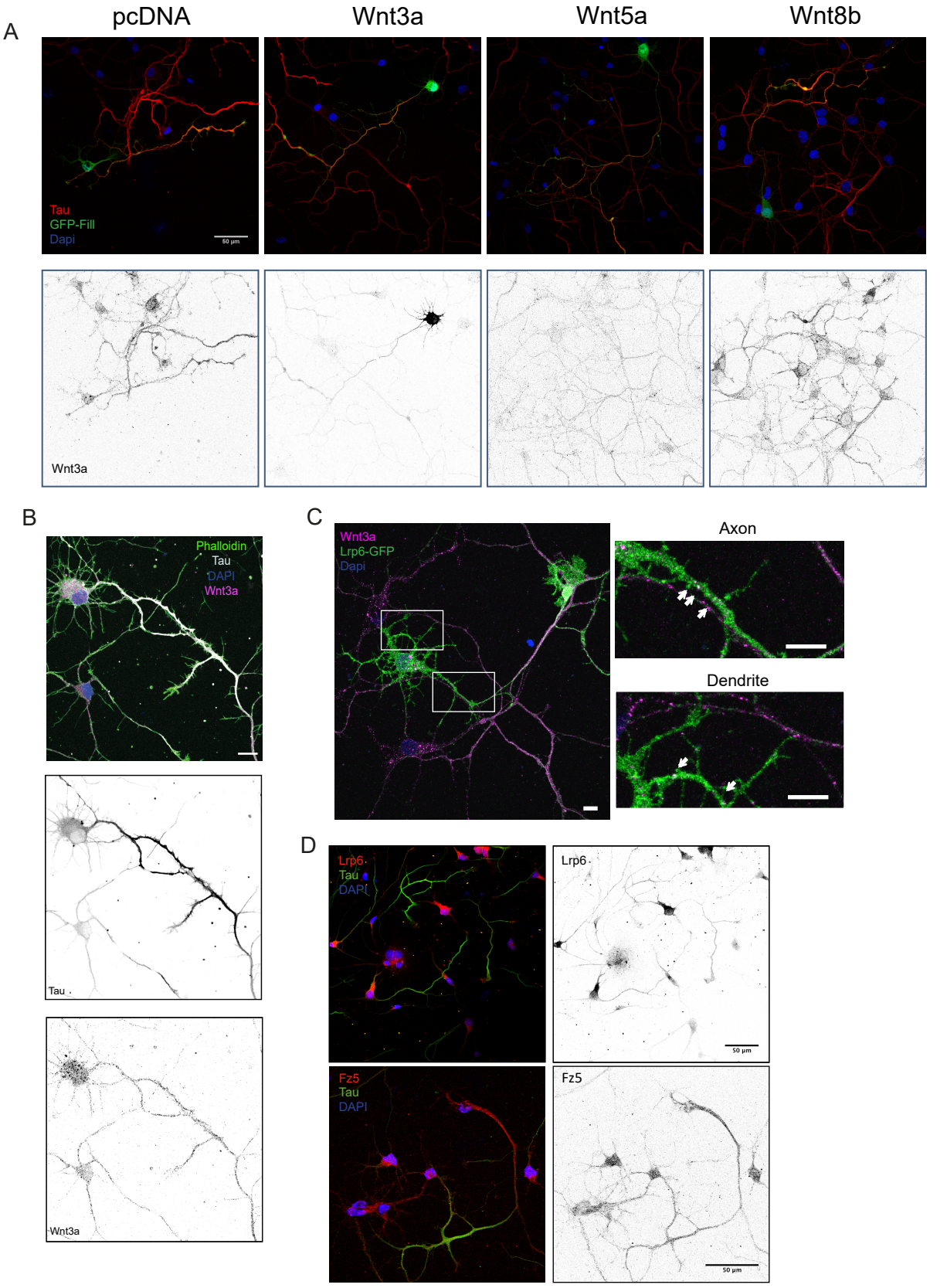

A
pcDNA
Wnt3a
Wnt5a
Wnt8b
Tau
GFP-Fill
Dapi
Wnt3a
B
Phalloidin
Tau
DAPI
Wnt3a
Tau
Wnt3a
C
Wnt3a
Lrp6-GFP
Dapi
Axon
Dendrite
D
Lrp6
Tau
DAPI
Lrp6
Fz5
Tau
DAPI
Fz5

Figure S1. Related to Figure 1. Overview of Wnt3a and LRP6 distribution in hippocampal neurons

A) Neurons were transfected with a GFP expression plasmid mixed with either pCDNA3, Wnt3a, Wnt5a or Wnt8B plasmids on Div0, then fixed and immunostained on Div2 against Tau and Wnt3a. Nuclei were counterstained with DAPI. (scale bar= 50µm).

B) Neurons were fixed at Div2, stained with antibodies against Wnt3a (magenta) and Tau (grey). Neurons were counterstained with Phalloidin (actin filaments; green) and DAPI (nuclei; blue) (left side: merged picture; right side: inverted gray scale channel of the endogenous Wnt3a expression and Tau (scale bar =10µm).

C) Neurons were transfected at Div3 with Lrp6-GFP, fixed at Div4 and stained for Wnt3a (magenta). Neurons were counterstained with DAPI (nuclei; blue). Overview picture of the Lrp6-GFP overexpressing neuron (left) from Figure 1B. The white boxes indicate the zoom-in of the axon and of a dendrite. Signalosome (co-localization between Wnt3a and Lrp6) are indicated with the white arrows.

D) Neurons were fixed at Div4, stained with antibodies against Lrp6 (upper panel) Fz5 (lower panel) and Tau (green). Neurons were counterstained DAPI (nuclei; blue) (left side: merged picture; right side: inverted gray scale channel of the endogenous Lrp6 and Tau.

Figure S2

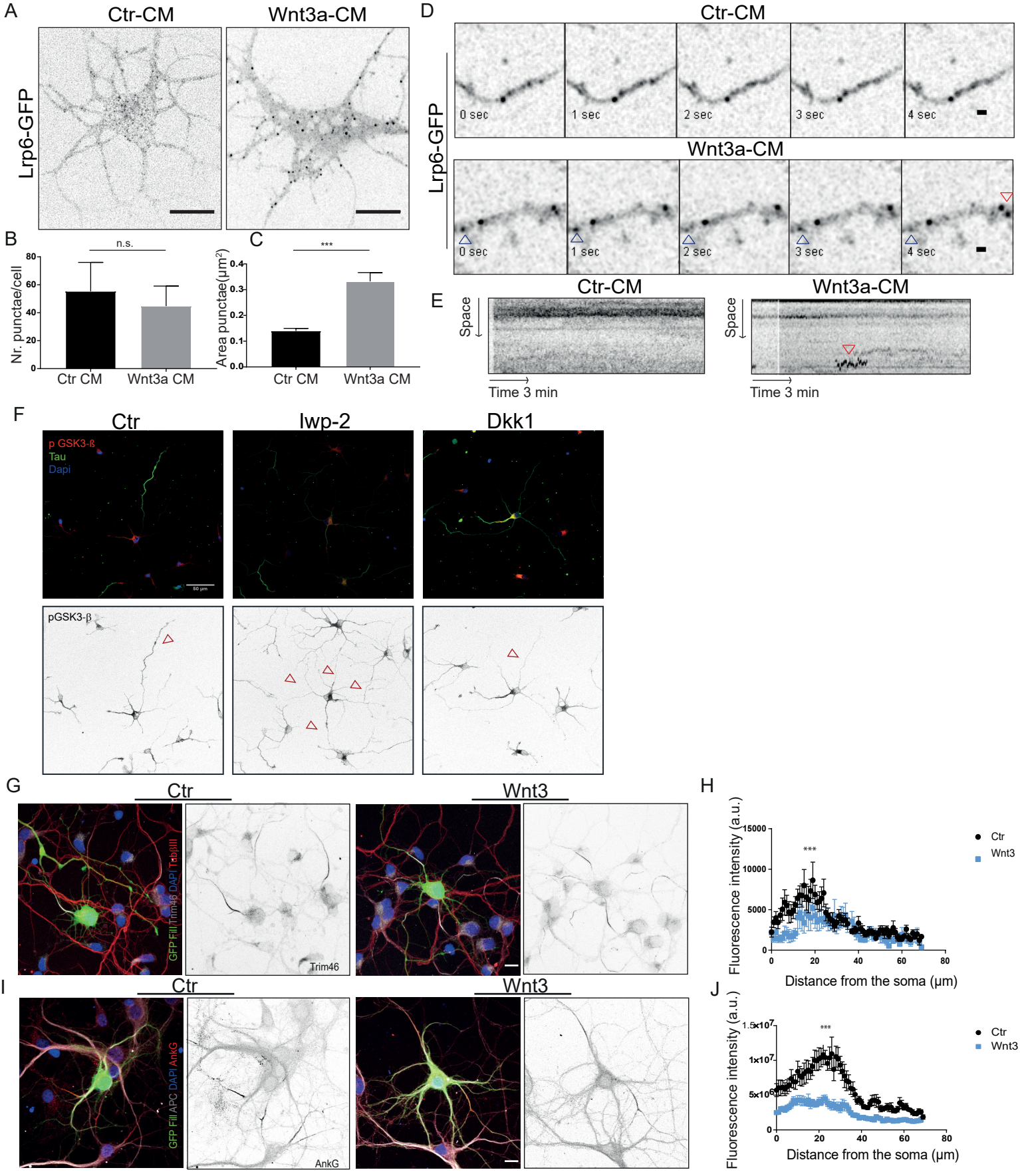

A
Ctr-CM
Wnt3a-CM
Lrp6-GFP
B
n.s.
Nr. punctae/cell
Ctr CM
Wnt3a CM
C
***
Area punctae(µm²)
Ctr CM
Wnt3a CM
D
Ctr-CM
Lrp6-GFP
0 sec
1 sec
2 sec
3 sec
4 sec
Wnt3a-CM
E
Ctr-CM
Wnt3a-CM
Space
Time 3 min
F
Ctr
Iwp-2
Dkk1
p GSK3-β
Tau
Dapi
pGSK3-β
G
Ctr
Wnt3
GFP Fill Trim46 DAPI Tubβ III
Trim46
H
Fluorescence intensity (a.u.)
Distance from the soma (µm)
Ctr
Wnt3
I
Ctr
Wnt3
GFP Fill APC DAPI AnkG
AnkG
J
Fluorescence intensity (a.u.)
Distance from the soma (µm)
Ctr
Wnt3

Figure S2. Related to Figure 1 and Figure 2. Wnt3a accumulation at the axon induces Lrp6 clustering and influences localization of AIS protein

A-E) Neurons were transfected at Div3 with Lrp6-GFP, at Div4 they were treated for 30 min with Wnt3a-CM or Ctr-CM and imaged at the spinning disk microscope.

A) Lrp6 clusters formation in neurons treated with Ctr-CM (left) and Wnt3a-CM (right) (scale bar =10µm).

B) Bar graph quantifying the number of puncta per cell (n=5) (n.s.=not significant).

C) Quantification of the area of the punctae (n=5) (***p<0.005).

D) Time lapse of Lrp6-cluster dynamics (1 frame per second) in the proximal axon of neuron treated with Ctr-CM (upper panel) and Wnt3a-CM (lower panel) (scale bar =2µm).

E) Kymograph of Lrp6 punctae in a Ctr-CM treated neuron (left) and Wnt3a-CM treated neuron (right).

F) Neurons were either left untreated or treated at Div0 with Iwp-2, or recombinant Dkk1 (100ng/ml). At Div2 neurons were fixed and stained for Tau, pGsk3β (inactive state) and counterstained with DAPI. Upper panel: Merged staining of Tau proteins (green), inactive Gsk3β (red) and nuclei (DAPI; blue). Lower panel: Inverted gray scale of intensity of the antibody staining against pGsk3β. Experimental conditions from left to right: untreated neurons, neurons treated with Iwp-2 and recombinant Dkk1. Pictures show representative results of at least 3 independent experiments (scale bar =50 µm). Red arrows heads indicate pGsk3β positive neurites.G-J) Neurons were co-transfected with a GFP-Fill (green) and an empty vector or Wnt3 at Div0.

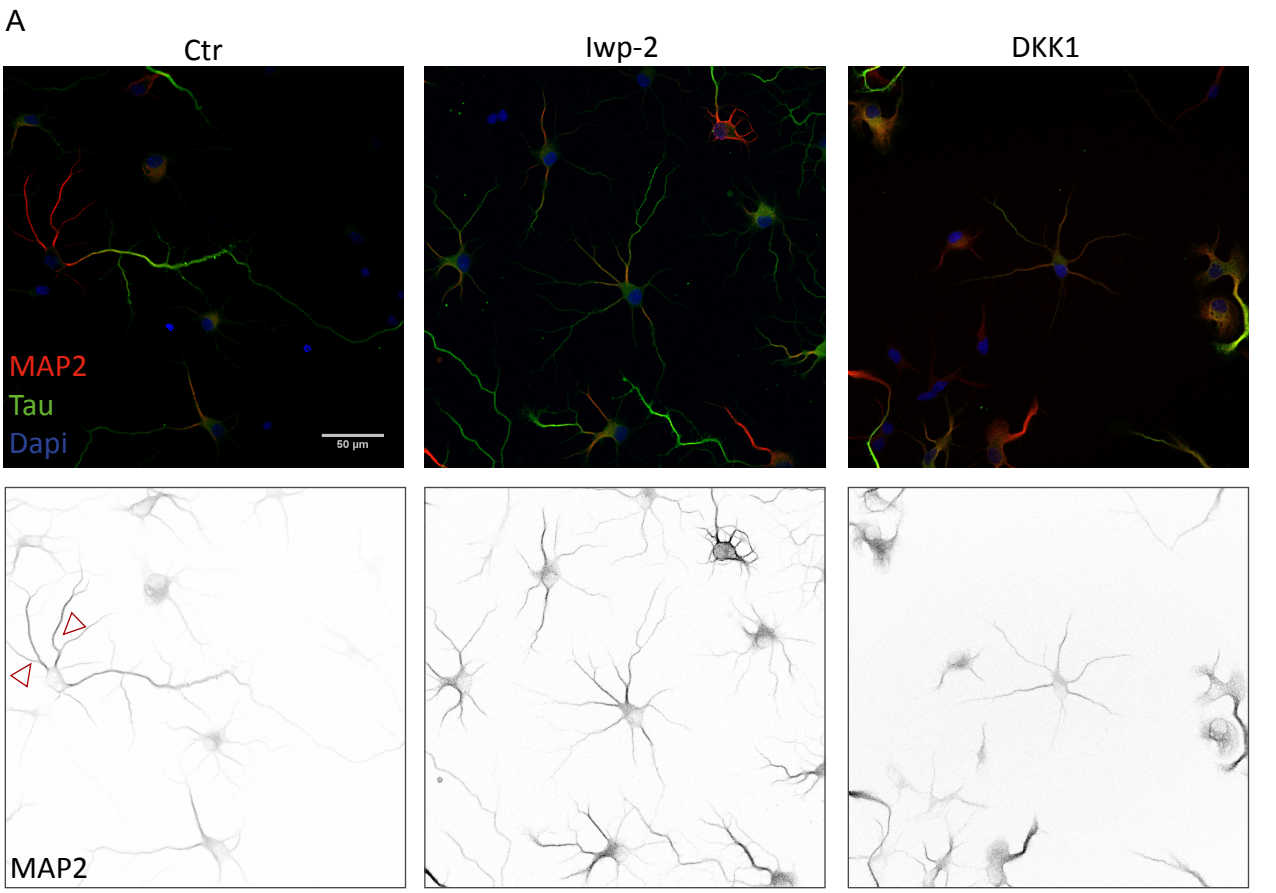
G) Transfected neurons were fixed at Div4. On the left control neurons stained for Trim46 (gray), TubulinβIII (red) and DAPI (blue)(merged picture left), inverted gray scale of Trim46 staining (right). On the right, Wnt3 transfected neurons stained for Trim46 (gray), Tubulin-βIII (red) and DAPI (blue) (merge picture left), inverted gray scale of Trim46 staining (right) (scale bar =10µm).

H) Quantification of the intensity profile of Trim46 in the axonal initial segment (n=12,***p<0.005).

I) Transfected neurons were fixed at Div8. On the left control neurons stained for AnkG (red), APC (gray) and DAPI (blue)(merge picture left), inverted gray scale of AnkG staining (right). On the right side Wnt3 transfected neurons stained for AnkG (red), APC (gray) and DAPI (blue) (merge picture left), inverted gray scale of AnkG staining (right) (scale bar =10µm).

J) Quantification of the intensity profile of AnkG in the axonal initial segment (n=12, ***p<0.005).

Figure S3


A
Ctr
Iwp-2
DKK1
MAP2
Tau
Dapi
50 µm
MAP2

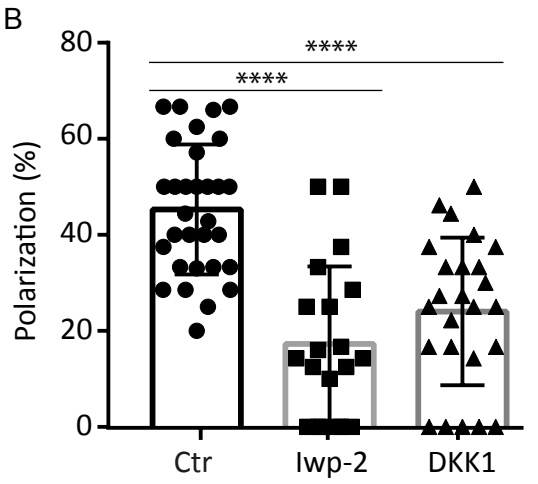

B
Polarization (%)
80
60
40
20
0
****
****
Ctr
Iwp-2
DKK1

Figure S3. Related to Figure 2. Inhibition of Wnt3a signaling induces mislocalization of Tau and MAP2

A) Neurons were either left untreated (left), treated with the inhibitor of Porcupine Iwp-2 (1µM) (middle) or the antagonistic inhibitor of the LRP6-Wnt3a interaction Dkk1 (100ng/ml) (right), respectively. Treatments were added on Div0 and the neurones were fixed on Div2. The neurons were stained for MAP2 (red), Tau (green) DAPI (blue). Below is the inverted gray scale of the intensity of the antibody staining against MAP2. The red arrows show the dendrites, enriched with MAP2 in the untreated condition. Pictures show representative results of at least 3 independent experiments for control and Iwp-2 and 2 for Dkk1 (scale bar = 50 µm).

B) graph representing the percentage of polarized neurons in each condition. Neurons with a correct localization of Tau in one of the neurites were counted as polarized. The pictures taken of each condition were quantified and normalized to the amount of healthy nuclei present in the picture and were taken randomly throughout the coverslip (****$p<0.001$). The graph shows the mean with standard deviation.

Figure S4

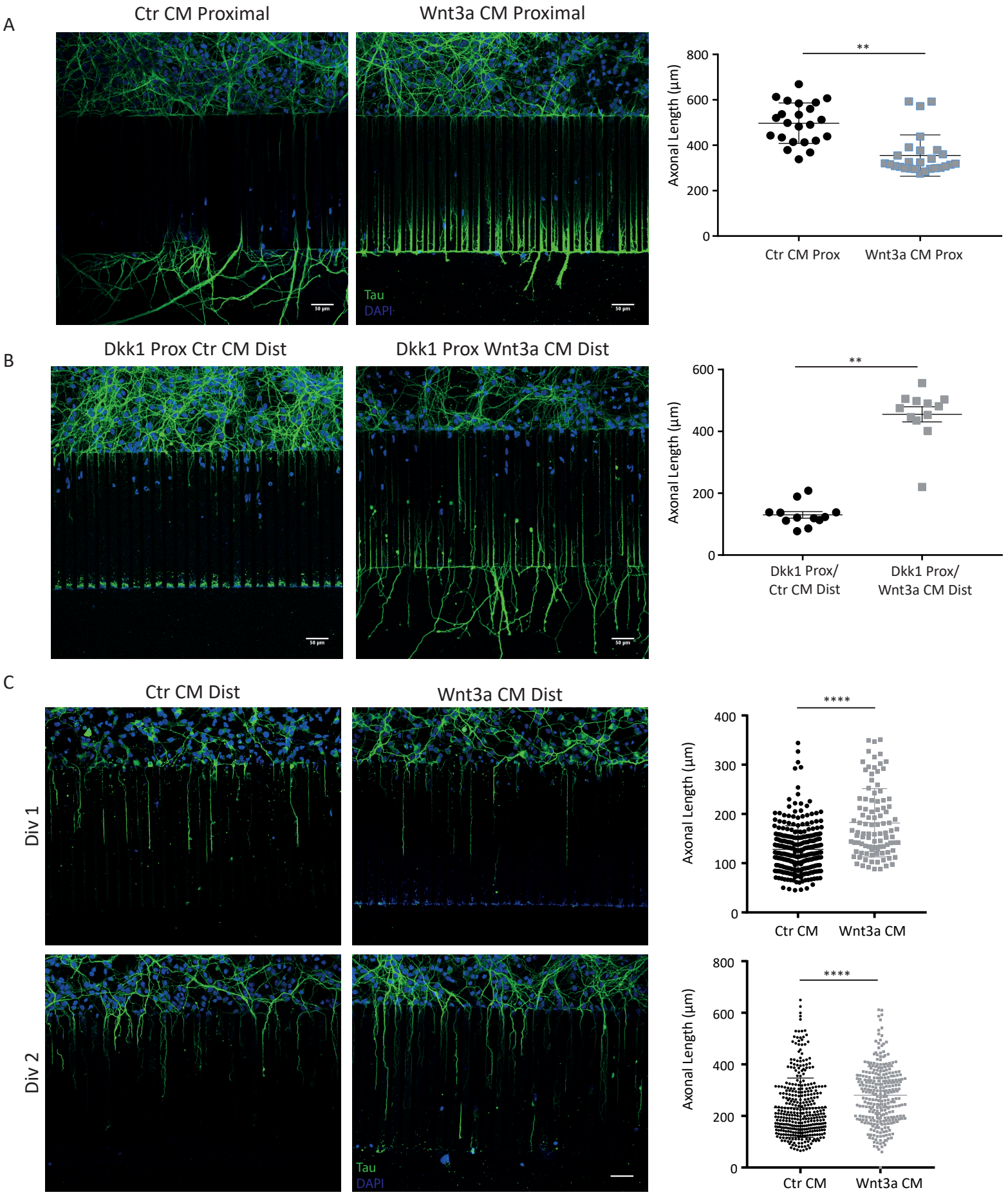

A
Ctr CM Proximal
Wnt3a CM Proximal
Tau
DAPI
Axonal Length (µm)
800
600
400
200
0
**
Ctr CM Prox
Wnt3a CM Prox
B
Dkk1 Prox Ctr CM Dist
Dkk1 Prox Wnt3a CM Dist
Axonal Length (µm)
600
400
200
0
**
Dkk1 Prox/
Ctr CM Dist
Dkk1 Prox/
Wnt3a CM Dist
C
Ctr CM Dist
Wnt3a CM Dist
Div 1
Div 2
Tau
DAPI
Axonal Length (µm)
400
300
200
100
0
****
Ctr CM
Wnt3a CM
Axonal Length (µm)
800
600
400
200
0
****
Ctr CM
Wnt3a CM

Figure S4. Related to Figure 3. Wnt3a acts as an early guidance cue

A) Div0 neurons were seeded in the proximal compartment of the microfluidic chamber and it was filled with either control-conditioned medium (left) or Wnt3a-conditioned medium (right). Axons were allowed to grow for 3 days. Afterwards, neurons were fixed, stained with antibodies against Tau and nuclei were counterstained with DAPI. Pictures show representative results of at least 3 independent experiments (scale bar =50 µm). Graph shows the average axonal length of the neurons (**$p<0.01$).

B) Neurons were seeded as in (A) and recombinant Dkk1 100 ng/ml was added, the distal compartment was filled with either control conditioned medium (left) or Wnt3a-conditioned medium (right). Axons were allowed to grow for 3 days. Afterwards, neurons were fixed and stained as in (A). Pictures show representative results of at least 3 independent experiments (scale bar =50 µm). Graph shows the average axonal length of the neurons) (**$p<0.01$).

C) Neurons were seeded as in (A) and the distal compartment was filled with Wnt3a or control-conditioned media. Neurons were fixed after 1 or 2 days (Div1 & Div2) then stained as in (A). Pictures show representative results of at least 3 independent experiments (scale bar =50 µm). Graph shows the average axonal length of the neurons) (n= number of axons measured. Div1 Ctr n=278, Div1 Wnt3a n=100, Div2 Ctr n=365, Div2 Wnt3a n=291. ****$p<0.0001$).

Figure S5

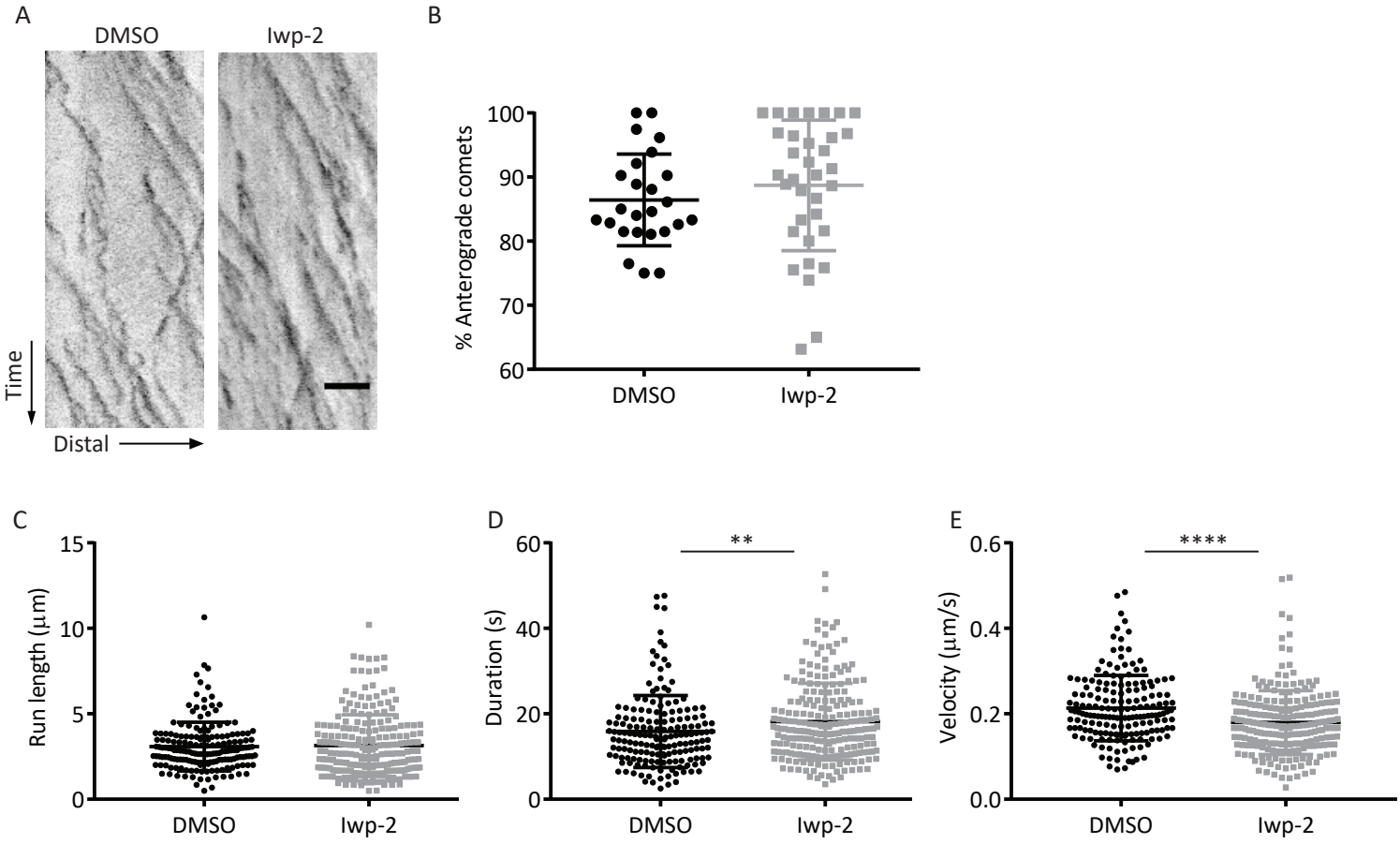

A
DMSO
Iwp-2
Time
Distal
B
% Anterograde comets
100
90
80
70
60
DMSO
Iwp-2
C
Run length (µm)
15
10
5
0
DMSO
Iwp-2
D
Duration (s)
60
40
20
0
**
DMSO
Iwp-2
E
Velocity (µm/s)
0.6
0.4
0.2
0.0
****
DMSO
Iwp-2

Figure S5. Related to Figure 4. Inhibition of Wnt secretion affects microtubule dynamics

A-E) Neurons were transfected at Div0 with mCherry-MACF43 (GFP-MT+ End) and imaged at the spinning disk microscope at Div1. Immediately after transfection, neurons were treated with Iwp-2 or DMSO 0.1% as control. Results are based on two independent experiments.

A) Representative kymographs of neurites in Iwp-2 and DMSO treated neurons. Time duration is 2 minutes. Scale bar= 5µm.

B) Average percentage of anterograde comets per all neurites measured (n= 25 & 34 for DMSO & Iwp-2 respectively).

C-E) A single neurite per neuron with the highest anterograde comet percentage was selected as the designated future axon. Run length, duration and velocity were calculated per comet (n= 174 & 224 comets for DMSO & Iwp-2, **$p<0.01$, ****$p<0.001$).

Transparent Methods

Ethic Statement

All experiments with animals were performed in compliance with the guidelines for the welfare of experimental animals issued by the Government of the Netherlands, and were approved by the Animal Ethical Review Committee (DEC) of Utrecht University.

Hippocampal neuron cultures, transfections and drug treatments

Primary hippocampal and cortical cultures were prepared from embryonic day 18 rat brains (both genders). Cells were plated on coverslips coated with poly-L-lysine (PLL, 37,5 mg/mL) and laminin (1,25 mg/mL) at a density of 100.000/well. Neurons were cultured in complete Neurobasal medium (NB) supplemented with 2% B27 (GIBCO), 0,5 mM glutamine (GIBCO), 15,6 mM glutamate (Sigma), and 1% penicillin/streptomycin (GIBCO) at 37 °C. For transfection 1,8 µg DNA/coverslip was incubated with 3,3 µl of Lipofectamine 2000 (Thermofisher, 11668019) in 200µl of NB for 30 min and added to the neuron in NB supplemented with 0,5 mM glutamine for 45 min. After this step, neurons were briefly washed and transferred back to the original medium. The following constructs have been used: β-tubulin-GFP (kind gift from Dr. P. Schätzle), GFP-Macf43 (Yau et al., 2014), Lrp6-GFP (kind gift from Dr. Gary Davidson), Wnt3a-FT (Farin et al., 2016), Wnt3 (Addgene, #35909).

Treatment of neurons with drugs and recombinant proteins

To induce multiple axons formation, neurons were treated with 10nM Taxol (Sigma-Aldrich, T7402) for 48h. The treatment was started at day *in vitro* (Div) 2 and neurons were fixed with 4% paraformaldehyde (PFA) at Div 4. To block Wnt secretion, neurons were treated on Div0 with 1 µM Iwp-2 (STEMCELL technologies, 72122,) and fixed with 4% PFA at indicated time points (see figure legends). To induce Wnt-signaling, neurons were stimulated with 40 ng/ml Wnt3a (R&D System, 1324-WN, time points are indicated in figure legends). Dkk1 (R&D System 5897-DK) was used at a concentration of 100 ng/ml.

Micropattern

Glass coverslips (VWR #631) were plasma activated (Harrick Plasma Cleaner) for 1 min at high intensity settings and then coated with Poly (L-Lysine)-graft-Poly (Ethylene Glycol) (PLL-PEG) (SuSoS, Switzerland) solution of 0.1 mg/ml in HEPES 10mM pH 7.4 for 30 minutes at room temperature (R.T.). Coverslips were washed in milliQ water, placed onto a photomask and then insolated under a deep UV lamp (Jelight, #42-220) for 4 min. The micropttern geometries were 10µm wide and 300µm in length (lines, Fig. 2F). Coverslips were

incubated for 2 hours at R.T. with 40µM BSA conjugated with Alexa Fluor 647 (ThermoFisher, #A34785) alone or in combination with 100 ng/ml of recombinant Wnt3a (R&D System, 1324-WN). After this step, coverslips were washed and coated with poly-L-Lysine (37.5 mg/ml). Neurons were plated at a density of 50.000 cells per coverslip. Neurons were manually categorized as described in Fig. 2E.

Microfluidic chamber (MFC)

MFC made of Sylgard 184© polydimethylsiloxane (PDMS) were cast and prepared as described previously, with minor alteration, based on the small-form factor design mold of two-compartment chamber (Taylor et al., 2005). Briefly, PDMS was mixed with curing agent at 10:1 w/w ratio and cast on Epoxy-resin molds. Cured PDMS devices were cut and four 5-mm wells were punched in the ends of the channels of both compartments. Devices were adhered to coverslips by plasma-cleaner activation (Harrick Plasma), then treated with pLL plus Laminin mixture to enable neuron attachment. Hippocampal neurons were plated at a density of 150.000/MFC in an initial volume of 4µl in one well of the MFC (proximal site). After 30 minutes of incubation 50 µl/microwell complete NB medium (supplemented as indicated for the neuron cultures) was added in the proximal compartment (on the same side where neurons were seeded). On the opposite side (distal compartment) complete NB medium was diluted 1:1 with either control- conditioned medium (Ctr-CM), Wnt3a-conditioned medium (Wnt3a-CM) or Wnt3a- conditioned media plus 100 ng/ml Dkk1 and added to the distal microwells (60 µl/microwell).

Wnt3a-conditioned media

L-cells (ATCC CRL-2648) were cultured in DMEM containing 1 g/l glucose (Life Technologies), supplemented with 10% FCS and P/S. All cells were maintained at 37 °C in 5% CO2. L-cells stably expressing and secreting Wnt3a or Wnt3a-FlagTag were cultured in the presence of 125 µg/ml Zeocine (Life Technologies) to obtain Wnt3a-conditioned medium (Shibamoto et al., 1998).

Immunofluorescence staining and imaging

Fixed cells were washed 3 times for 5 min in PBS, permeabilized in 0.25% TritonX-100, incubated for an hour at R.T. with BSA 2% and after with the primary-antibody in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM phosphate buffer, pH 7.4) overnight at 4°C. After 3 times washing with PBS, cells were incubated with secondary-antibodies in GDB buffer for an hour at R.T. After washing, coverslips were mounted in Vectashield), Vectashield antifade mounting medium with DAPI (Vectorlabs, H-1200). The primary antibodies used were: rabbit anti-TRIM46 [37] rabbit anti-β-III tubulin (Covance, AB_291637), mouse anti-β-III tubulin (Sigma AB_082M4845), mouse anti- dephosphorylated Tau (clone PC1C6,

Chemicon, AB_94855), rabbit anti-MAP2 (Cell Signaling, CST_4542), mouse anti-AnkirinG (Zymed, Life technologies AB_33-8800), rabbit anti-Wnt3 (Abcam AB_28472), rabbit anti β-catenin (Abcam AB_32572), rabbit anti-Frizzled5 (Abcam AB_75234), rabbit anti-LRP6 (Abcam AB_66156), rabbit anti-phospho Ser9 Gsk3 β (Cell Signaling, AB_9323). Secondary antibodies: anti-mouse Alexa488 (Life Technologies, AB_2534088), anti-rabbit Alexa488 (Life Technologies, A11034, RRID: AB_2576217), anti-mouse Alexa568 (Life Technologies A11031, RRID: AB_144696), anti- rabbit Alexa568 (Life Technologies, A11036, RRID: AB_10563566), anti-mouse Alexa647 (Life Technologies A21236, RRID: AB_2535805), anti-rabbit Alexa647 (Life Technologies, A21245, RRID: AB_2535813). Neurons were imaged with a LSM700 confocal laser-scanning microscope (Zeiss) with a Plan-Apochromat 63x NA 1.40 oil DIC, EC Plan-Neofluar 40x NA1.30 Oil DIC and a Plan-Apochromat 20x NA 0.8 objective. Each confocal image was a z series of 5–10 images, each averaged 4 times, covering the entire region of interested from top to bottom. Maximum projections were done from the resulting z stack. For fluorescence intensity comparison, settings were kept the same for all conditions.

Live-cell imaging

Live-cell imaging experiments were performed in an inverted microscope Nikon Eclipse Ti-E (Nikon), equipped with a Plan Apo VC 100x NA 1.40 oil and a Plan Apo VC 60x NA 1.40 oil objective (Nikon), a Yokogawa CSU-X1-A1 spinning disk confocal unit (Roper Scientific), a Photometrics Evolve 512 EMCCD camera (Roper Scientific) and an incubation chamber (Tokai Hit) mounted on a motorized XYZ stage (Applied Scientific Instrumentation) which were all controlled using MetaMorph (Molecular e5 Neuron 94, 809–825.e1–e7, May 17, 2017). Quantification of moving particles length (length of the comet run (C) multiplied by sin (α) where α is 90 minus the angle created by the comet truck and a horizontal line), duration (C* cos(α)) , and velocity (tan (α)) of dynamic microtubules (labeled with GFP-MACF43), were quantified from kymographs of proximal axons created using the Kymoreslicewide plug- in under Fiji (https://github.com/ekatrukha/KymoResliceWide) under ImageJ (NIH, https://imagej.nih.gov/ij/). Lrp6 punctae counting and area measurements were performed using the Analyze Particle function of Image J. Axonal length was calculated by drawing in ImageJ a segmented line along the axon starting from the beginning of the channel to the end of the field of view.

Quantification and Statistical Analysis

All statistical details of experiments, including the definitions and exact values of n, and statistical tests performed, can be found in Figures and Figure Legends. Data processing and statistical analysis were done in Excel and GraphPad Prism (GraphPad Software). Significance

was defined as: ns-not significant, $^{*}p < 0.05$ $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistical analysis: Unpaired t test.

Supplemental References

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