Supplementary Material

for

Live imaging of excitable axonal microdomains in ankyrin-G-GFP mice

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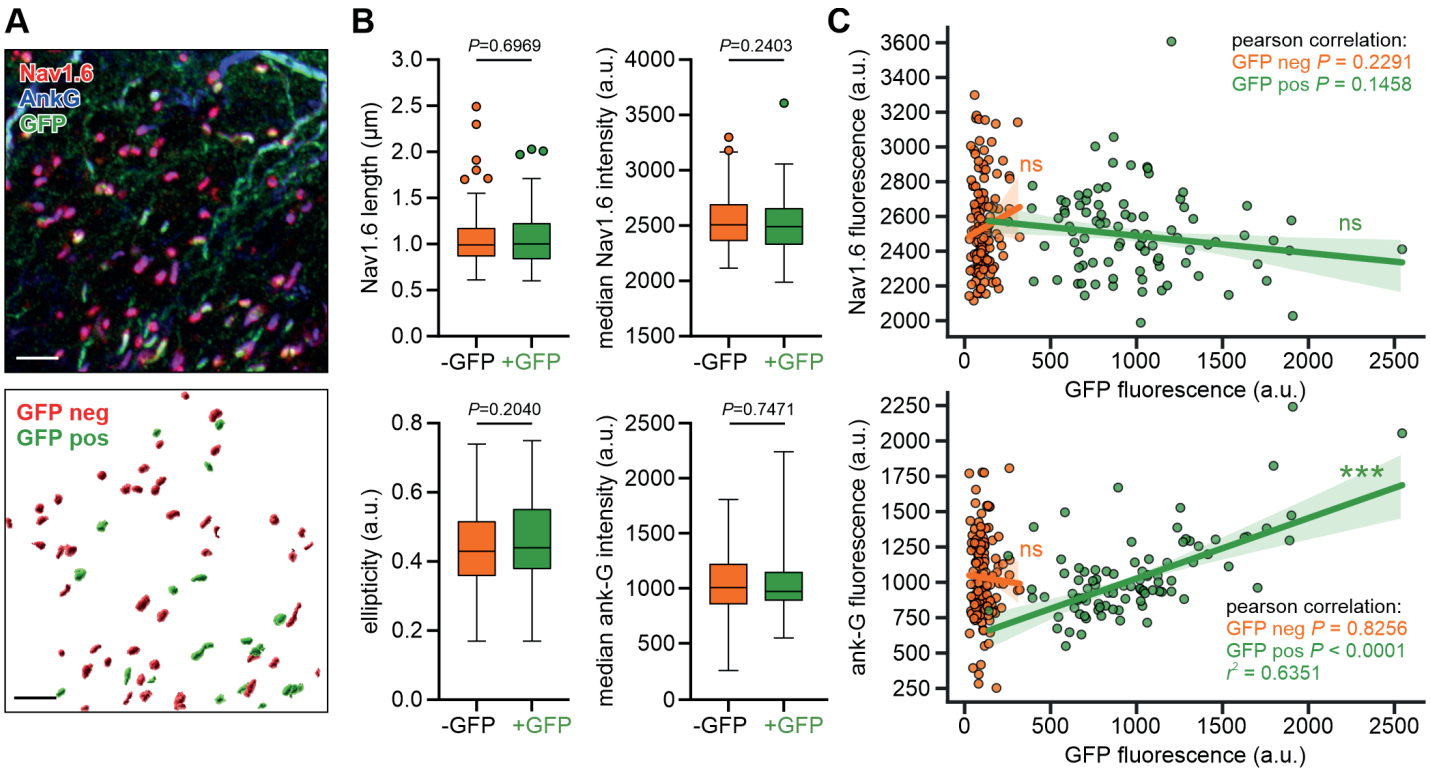
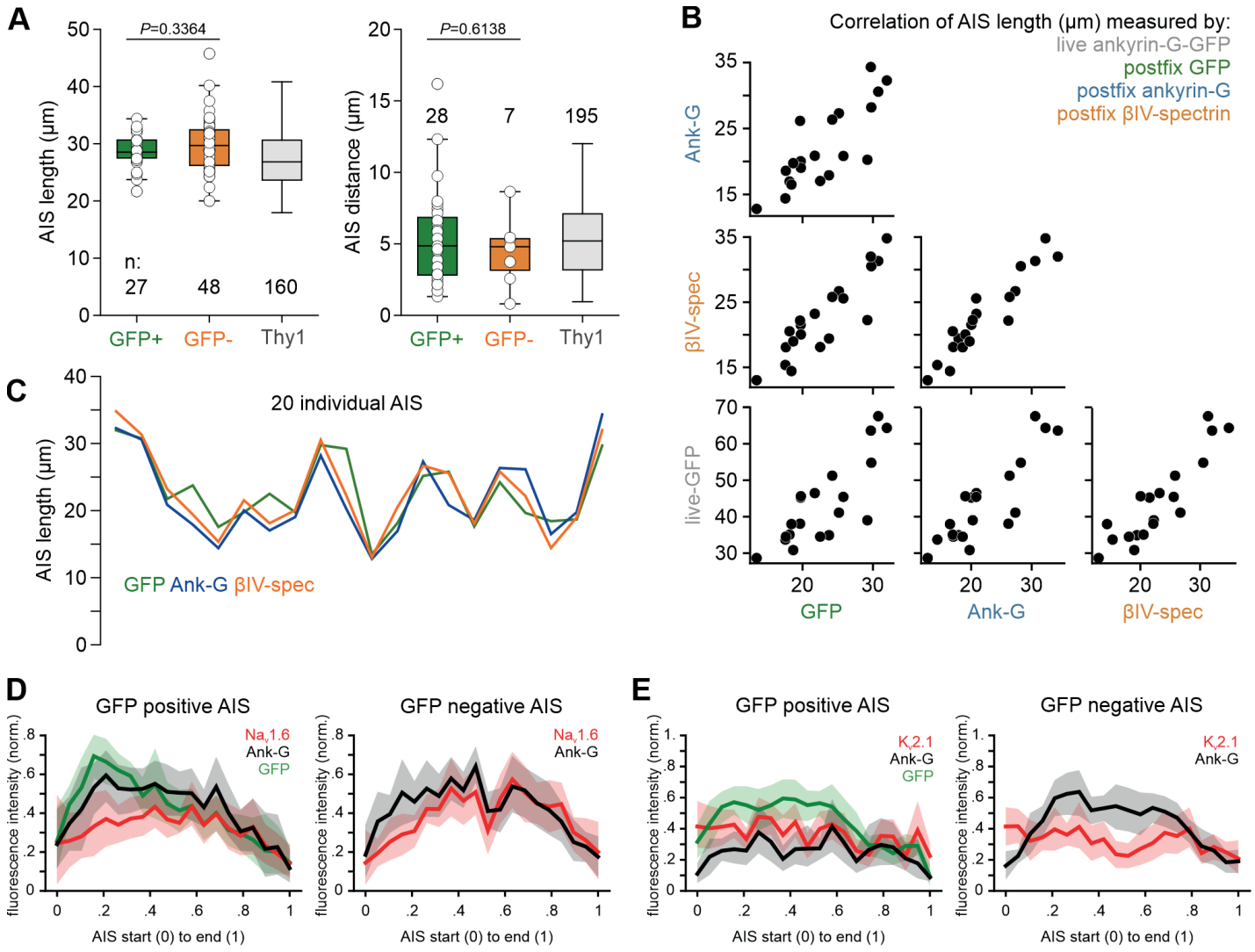


Supplementary Figure S1: Ank-G-GFP activation and expression in nodes of Ranvier do not alter node morphology

A Top panel shows a cryosection of neocortical white matter from an ankyrin-G-GFP x CaMKIIa-Cre mouse, with GFP⁺ and GFP⁻ nodes of Ranvier (noR; see Fig. 3A for details). The bottom panel shows an automated 3D reconstruction of noR using Imaris. Nodes were identified via the ankyrin-G channel, classified by the GFP channel, and properties were analyzed in $Na_v1.6$ and ankyrin-G channels. All steps were automated within the Imaris software. Scale bar = 5 µm. **B** Nodes that are GFP⁺ and GFP⁻ show no differences regarding their length, ellipticity, and median fluorescence intensity of ankyrin-G or $Na_v1.6$ signals (n = 141 GFP⁻ nodes, 91 GFP⁺ nodes, 1 animal, Mann-Whitney U test). **C** *Top* panel: The fluorescence intensity of the sodium channel $Na_v1.6$ did not correlate with ankyrin-G-GFP fluorescence intensity, indicating unchanged levels of sodium channels. *Bottom* panel: The intensity of GFP fluorescence correlates positively with the levels of all ankyrin-G in GFP⁺ but not GFP⁻ nodes (n as in B; Pearson correlation details in the graph). This demonstrates that ankyrin-G-GFP does not change the $Na_v1.6$ channel fluorescence intensity and provides a reliable predictor of ankyrin-G levels, though we do not exclude the possibility that native unlabeled ankyrin-G remains in the nodes to some degree.



Supplementary Figure S2: AIS length, position, and molecular composition remains intact after ankyrin-G-GFP expression

A Measurements of length and position of AIS in CA1 pyramidal neurons that are either GFP positive (green) or negative (orange). The inclusion of GFP into the ankyrin-G gene does not significantly change AIS length (*left* panel) or distance to the soma (*right* panel). Sample preparation was conducted as outlined in Fig. 7 and AIS measurements were based on the βIV-spectrin signal (n = 3 animals). An independent Thy1-GFP control line (grey, n = 3 animals) was used as an additional control. The number of individual AIS (white circles) and P values are given within the graph (t-test for AIS length; Mann – Whitney U-test for AIS distance). **B** Correlation of AIS length measured live via the ankyrin-G-GFP signal in a patch clamp chamber (grey) and post-fixation using antibodies against GFP, ankyrin-G, and βIV-spectrin in CA1 pyramidal neurons (sample preparation as in Fig. 7, n = 20 AIS, 1 animal). **C** Alternative visualization of data from panel B (post-fixation). Measurements for AIS signals using antibodies against GFP, ankyrin-G, and βIV-spectrin show comparable lengths. **D** Immunosignals from Na$_v$1.6 channels retain their fluorescence intensity across the AIS after the expression of ankyrin-G-GFP. AIS length was normalized from start to end of the ankyrin-G signal and fluorescence intensity from lowest to highest. Tissue preparation as described in Fig. 4A (n = 10 GFP$^+$ and 10 GFP$^-$ AIS). **E** Line plots of K$_v$2.1 and GFP fluorescence intensities along the AIS signal indicate no change in K$_v$2.1 expression. Normalization as in panel D. Tissue preparation as described in Fig. 4B (n = 10 GFP$^+$ and 10 GFP$^-$ AIS).

Supplementary Table 1 Summary of statistics for passive and active properties (related to Fig. 7)

stats	wildtype				AnkG-GFP				AnkG-GFP+Cre				ANOVA	-Cre / +Cre
	N	mean	median	SD	N	mean	median	SD	N	mean	median	SD	p(A) / *p(KW)*	ttest / *M-Whit*
RMP	28	-63.29	-63.56	3.78	22	-62.57	-63.43	4.62	42	-64.14	-63.32	4.17	0.3441	0.1733
Rinput	28	268.60	251.50	95.51	23	226.10	203.70	89.55	42	253.00	234.40	95.75	*0.0952*	0.1634
Ih sag	28	22.25	22.01	8.67	23	22.84	23.30	8.64	42	25.06	28.05	9.91	*0.2529*	*0.3469*
rheobase	28	52.16	44.86	30.90	23	70.86	69.81	36.43	41	69.31	69.81	37.54	*0.0654*	*0.9917*
ISI (ratio 1/6)	27	2.55	2.32	1.00	22	2.39	2.11	0.68	42	1.98	1.86	0.47	*0.0038*	*0.0123*
maxAP	27	12.04	11.00	2.59	22	9.77	10.00	2.05	42	10.52	10.00	2.48	0.0044	0.2278
IhalfmaxAP	27	102.40	96.82	39.97	22	122.20	120.10	32.56	42	122.90	116.30	46.33	*0.0576*	*0.6471*
AP thesh	28	-39.00	-38.45	2.75	23	-38.30	-38.70	3.75	42	-36.91	-36.54	3.41	*0.0163*	*0.0457*
AP amp.	28	84.98	86.78	10.90	23	88.85	90.03	7.73	42	90.29	91.51	6.92	0.0396	*0.4537*
AP hw	28	2.05	2.04	0.26	23	1.91	1.88	0.21	42	1.94	1.91	0.21	*0.0546*	*0.5371*
AP rise	28	0.54	0.54	0.12	23	0.50	0.49	0.07	42	0.46	0.45	0.08	*0.0013*	*0.0103*
AP decay	28	2.19	2.19	0.41	23	2.07	2.03	0.31	42	2.19	2.20	0.27	*0.2392*	0.1174
EPSP amp.	27	0.59	0.59	0.08	22	0.59	0.59	0.10	42	0.61	0.58	0.12	*0.8246*	*0.5555*
EPSP frequ.	27	0.47	0.31	0.61	22	0.49	0.32	0.50	42	0.40	0.25	0.41	*0.6986*	*0.3858*

multiple comparisons:		
significant pairs:	ISI (ratio 1/6)	Ank-G-GFP+Cre is different from both others
	maxAP	wildtype is different from both Ank-G-GFP groups
	AP thesh	wildtype vs. ankg□GFP+cre
	AP amp.	wildtype is different to ankG□GFP+Cre
	AP rise	wildtype vs ankG□GFP+Cre

Supplementary Table 2 Summary of Cre viruses and Cre driver lines

Neuron population	Cre virus / line	Common name	Source, strain
Unspecific	FUW-nGFP::Cre (lentiviral vector), CMV promotor	nGFP-Cre, NA	Gift from the Südhof lab, Stanford University, CA, USA
Unspecific	FUW-nGFP:: ΔCre (lentiviral vector), CMV promotor	nGFP-ΔCre, NA	Gift from the Südhof lab, Stanford University, CA, USA
Excitatory neurons	pENN.AAV.CamKII 0.4.Cre.SV40	CaMKII-Cre	Addgene viral prep #105558-AAV5, Lot: v7050
Neurons (unspecific)	ENN.AAV.hSyn.Cre.WPRE.hGH	Synapsin-Cre	Addgene viral prep #105553-AAV1, Lot: v75882
Inhibitory neurons	AAV1-hDlx-Flex-dTomato-Fishell_7	hDlx-tdTomato	Addgene viral prep #83894-AAV1
Neurons (unspecific)	pAAV-hSyn-Cre-P2A-dTomato	Synapsin-Cre-tdTomato	Addgene viral prep 107738-AAVrg, Lot: v75881
Excitatory neurons	B6.Cg-Tg(Camk2a-Cre)T29-1Stl/J	T29-1 IMSR_JAX:005359	The Jackson Laboratory Strain # 005359
Parvalbumin-pos. interneurons with tdTomato	B6;129P2-Pvalb$^{tm1(Cre)Arbr}$/J crossed with B6.Cg-GT(ROSA)26Sor$^{tm14(CAG-tdTomato)/Hze}$/J	B6 PV^{Cre} IMSR_JAX:017320 Ai14 IMSR_JAX:007914	The Jackson Laboratory Strain # 017320 Strain # 007914
Unspecific	AAV-retro/2-hSyn1-mCherry-iCre-WRPE-hGHp(A)	Retro-mCherry-Cre	University of Zürich, Viral Vector Facility, v230

Supplementary Table 3 Specification of primary and secondary antibodies (catalog number, working dilution, previously conducted controls, sources, Research Resource Identification Portal (RRID) code and references where available).

Primary Antibody Clone/type	Dilution	Reported controls				Source & Catalog Number RRID or other reference
		KO	IF	IP	WB	
Ankyrin-G (rb)	1:500		X		X	Santa Cruz Biotechnology, Heidelberg, Germany; sc-28561 AB_ 633909
Ankyrin-G (gp)	1:1000		X		X	Synaptic Systems, Göttingen, Germany 386 005 [1]
Ankyrin-G (ms) N106/36	1:500	X	X		X	UC Davis/NIH NeuroMab Facility, CA, USA; 73-146 AB_2315803
ank-G C-terminus	1:200		X			Santa Cruz Biotechnology, Heidelberg, Germany; sc-28561; discontinued
ßIV-spectrin (rb)	1:1000	X	X		X	Self-made [2-4]
Caspr (ms) K65/35	1:500	X	X	X	X	UC Davis/NIH NeuroMab Facility, CA, USA; 75-001 AB_2083496
FGF14 (ms) N56/21	1:500	X	X	X	X	UC Davis/NIH NeuroMab Facility, CA, USA; 75-096 AB_2104060
GFP (ch)	1:1000		X			Acris Antibodies GmbH, Hiddenhausen, Germany; AP20142PU-N AB_10756183
Iba1 (rb)	1:2000		X			Wako, Neuss, Germany; 019-19741 AB_839504
$K_v1.2$ (ms) K14/16	1:200	X	X	X	X	UC Davis/NIH NeuroMab Facility, CA, USA; 73-008 AB_2296313
$K_v2.1$ (ms) K89/34	1:1000	X	X		X	UC Davis/NIH NeuroMab Facility, CA, USA; 75-014-020 AB_2877280
Nav1.6 (rb)	1:2000		X		X	Alomone Labs, Jerusalem, Israel ASC-009 AB_2040202
NeuN (ms) *A60*	1:500		X		X	Millipore, Temecula, CA, USA; MAB377 AB_2314889
NeuN (gp)	1:2000		X			Synaptic Systems, Göttingen, Germany 266 004 AB_2619988
Parvalbumin (rb)	1:500		X		X	Swant Inc., Marly, Switzerland PV27 AB_2631173
Synaptopodin (gp)	1:500		X		X	Synaptic Systems, Göttingen, Germany 163 004 AB_10549419
TRIM46 (ms) *SMP14* *(aka MDM2)*	1:500		X		X	Santa Cruz Biotechnology, Heidelberg, Germany; sc-965 AB_627920
vGAT (ch)	1:1000		X			Synaptic Systems, Göttingen, Germany 131 006 AB_2619820

KO absence of immunosignal in knock out animals, *IF* immunofluorescence, *IP* immuno-precipitation, *WB* western blot, *rb* rabbit, *ms* mouse, *gp* guinea pig, *ch* chicken

Supplementary Table 3 (continued) Specification of primary and secondary antibodies (catalog number, working dilution, previously conducted controls, sources, Research Resource Identification Portal (RRID) code and references where available).

Secondary Antibody Clone/type	Dilution	Reported controls				Source & Catalog Number RRID or other reference
		KO	IF	IP	WB	
FluoTag-X4-anti GFP-StarRED 1H1/1B2	1:500					NanoTag Technologies, Göttingen, Germany; N0304 AB_2744631
gt anti mouse abberior STAR 580	1:100					Abberior GmbH, Göttingen, Germany; ST580-1001 AB_2923543
gt anti rabbit abberior STAR 580	1:100					Abberior GmbH, Göttingen, Germany; ST580-1002 AB_2910107
gt anti ch Alexa Fluor 488	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-11039 AB_ 42924
gt anti ms Alexa Fluor 568	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-11004 AB_143162
gt anti rb Alexa Fluor 568	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-21069 AB_141416
gt anti gp Alexa Fluor 568	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-11075 AB_ 141954
gt anti ch Alexa Fluor 568	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-11041 AB_2534098
gt anti ms Alexa Fluor 594	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-21125 AB_141593
gt anti rb Alexa Fluor 594	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-11012 AB_141359
gt anti ms Alexa Fluor 647	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A AB_2535804-21235
gt anti rb Alexa Fluor 647	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-21244 AB_2535812
gt anti gp Alexa Fluor 647	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-21450 AB_2735091
gt anti ch Alexa Fluor 647	1:500					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; A-21449 AB_2535866
Alexa Streptavidin 568	1:1000					Molecular Probes, Thermo Fisher, Karlsruhe, Germany; *S11226* AB_2315774

KO absence of immunosignal in knock out animals, *IF* immunofluorescence, *IP* immuno-precipitation, *WB* western blot, *rb* rabbit, *ms* mouse, *gp* guinea pig, *ch* chicken

Supplementary Table 4 Summary of fixation and blocking reagents for all immunofluorescence experiments.

Sample	Fixation Time / DIV	Post Fix	Blocking buffer Incubation buffer
Isolated hippocampal neurons	4% PFA, 15 min DIV 19	No	**B:** 1% BSA in PBS. Prior to blocking, quench in PBS with 100 mM glycine, 100 mM ammonium chloride (5 min) and application of 0.1% Triton X-100 for 5 min **I:** PBS
Hippocampal OTC	4% PFA, 30 min	No	**B:** 0.1% Triton X-100, 1% BSA, 0.2% fish skin gelatine, in 1 x PBS **I:** same
Ex vivo acute slices	2% PFA, 90 min	No	**B:** 0.3% Triton X-100, 5% normal goat serum in 1 x PBS **I:** 0.2% Triton X-100, 1% normal goat serum in 1 x PBS
Whole mount retina	4% PFA, 15 min >P55	No	**B:** 0.5% Triton X-100, 0.2% BSA, 0.02% sodium azide in 1x PBS **I:** 1% Triton X-100, 10% FCS and 0.02% sodium azide in 1x PBS
Cryosections	Perfusion, 15 min 2% PFA (for ion channels) 4% PFA (for all others) >P28	No	**B:** 1% BSA, 0.2% fish skin gelatine, 0.1% Triton X-100 in 1 x PBS **I:** same

PFA paraformaldehyde, *min* minutes, *DIV* days in vitro, *BSA* bovine serum albumin, *PBS* phosphate buffered saline, *FCS* fetal calf serum

References for Supplements

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2. Gutzmann, A., et al., A period of structural plasticity at the axon initial segment in developing visual cortex. Front Neuroanat, 2014. 8: p. 11.
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4. Höfflin, F., et al., Heterogeneity of the Axon Initial Segment in Interneurons and Pyramidal Cells of Rodent Visual Cortex. Front Cell Neurosci, 2017. 11: p. 332.