Supplementary information

Threat intensity shapes cortical engram architecture supporting remote memory retrieval

Authors

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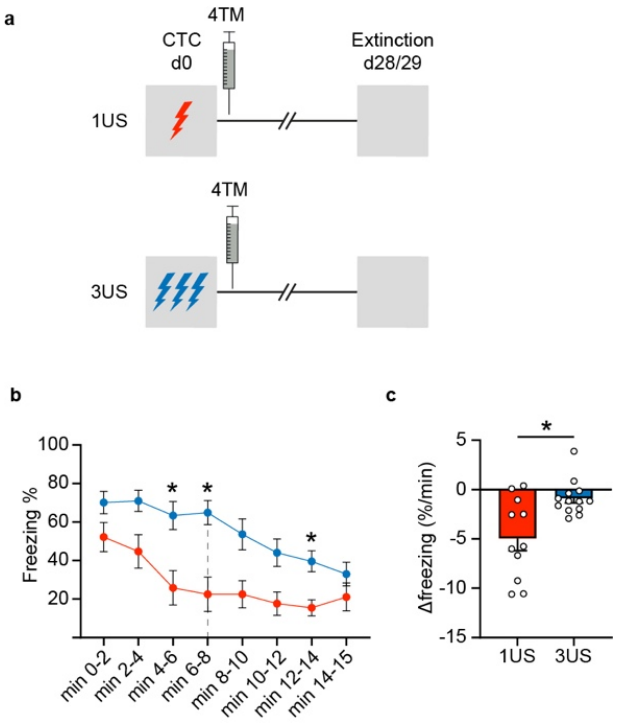
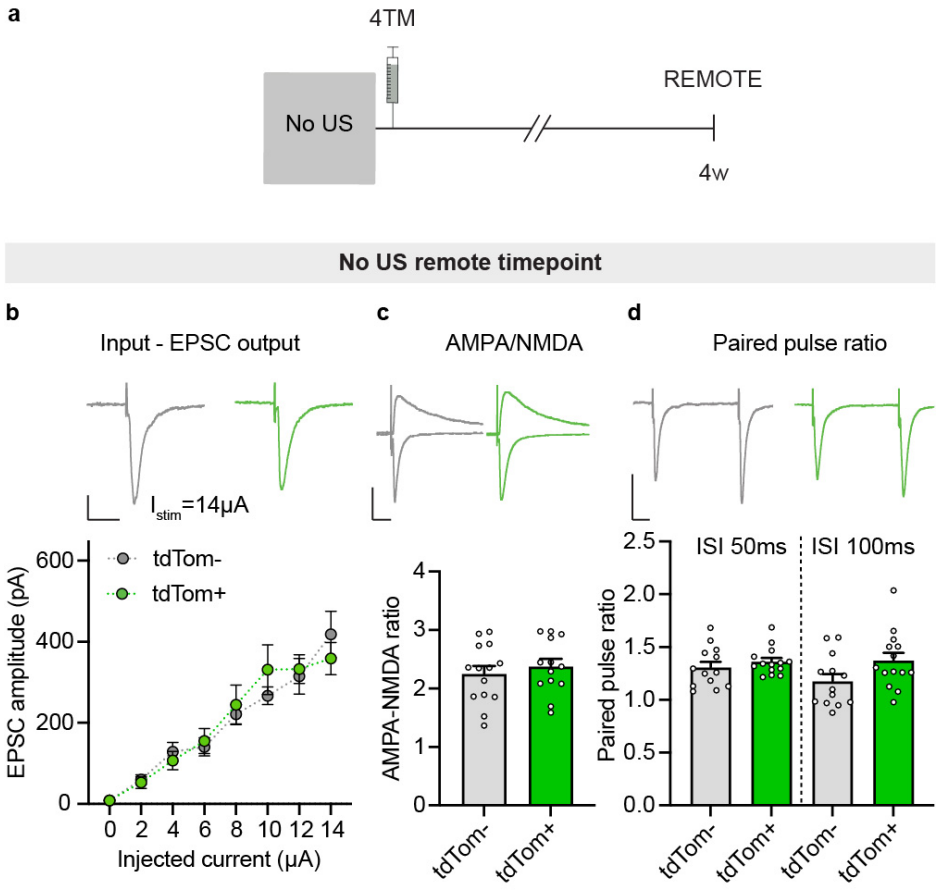
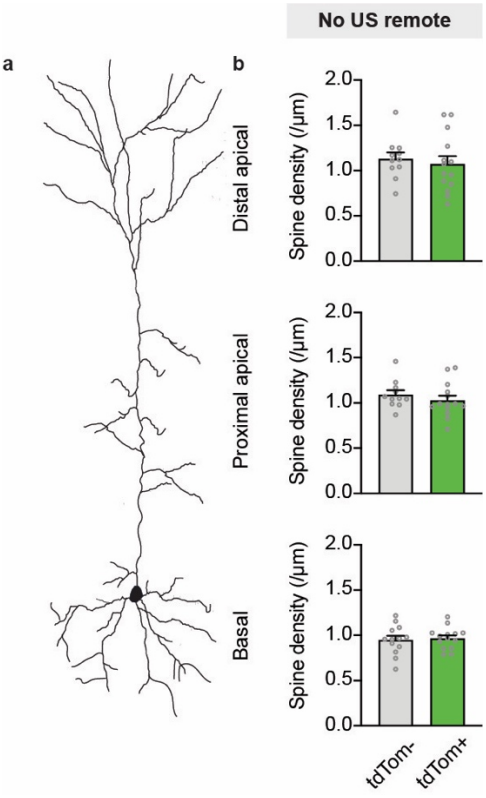


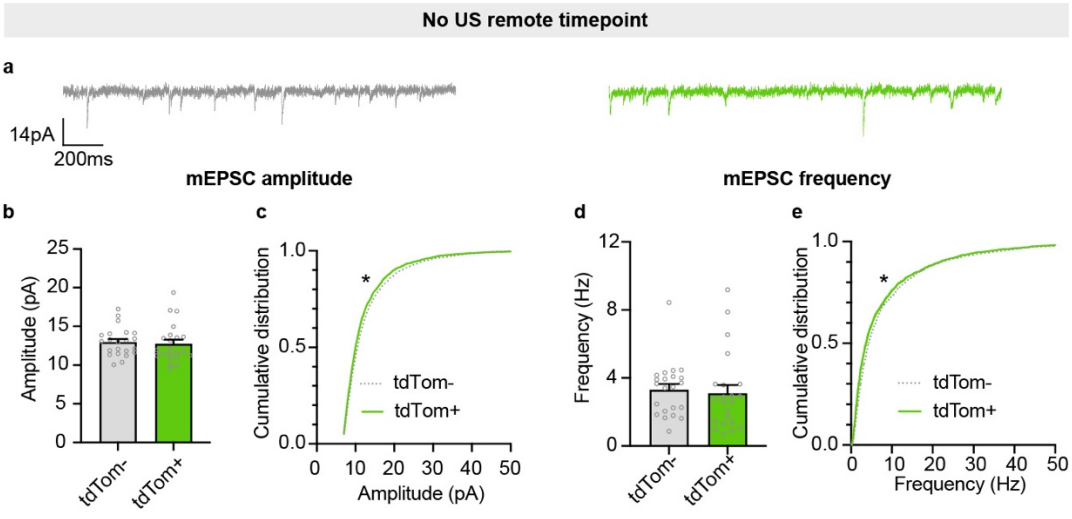
Supplementary Figure 1. Strong threat memory is more resilient to extinction learning than mild threat memory. a Experimental design. Mice were exposed to either 1US or 3US CTC. At day 28/29 after CTC, mice were alowed to freely explore the conditioning context for 15 min in absence of foot-shocks. **b** Freezing levels did not differ significantly during the first 2 min of the session, but subsequently extinguished faster in the 1US group. Two way RM ANOVA: Time x Group: $F_{7,154} = 2.2$, $p = 0.028$, Group: $F_{1,22} = 12.3$, $p = 0.002$, Time: $F_{7,154} = 16.2$, $p < 0.0001$). Post-hoc. Bonferroni test revealed a difference between groups at min 4-6 (*$p = 0.032$), min 6-8 (*$p = 0.008$) and min 12-14 (*$p = 0.016$). 1US: n = 11, 3US: n = 13. Grey dashed line indicates the middle of the extinction session. **c** Given that 1US mice already reached stable low freezing levels halfway the session, we compared the change in freezing between min 0-2 and min 6-8 by calculating the slope. This also confirmed that 1US mice extinguished faster than 3US mice (Mann-Whitney U test, U = 34, $p = 0.030$). Bar graphs show mean + s.e.m. Individual data points are mice. Source data are provided as a Source Data file.



Supplementary Figure 2. Evoked excitatory synaptic transmission is unaltered in mice that undergo context exposure alone. a Experimental design. PL neurons were tagged after exposure to the conditioning context in absence of a foot shock (No US) and 28-31 days later (remote timepoint) eEPSCs were recorded from neighboring tdTom$^+$ and tdTom$^-$ PNs in layer 5 of the PL. **b** Top: representative eEPSC in response to 14 µA stimulation. The eEPSC amplitude in relation to the stimulation intensity did not differ between tdTom$^-$ (grey) and tdTom$^+$ (green) neurons (Mixed-effects model: Population x Stimulation interaction: $F_{7,180} = 0.9$, $p = 0.52$; Population: $F_{1,27} = 0.1$, $p = 0.76$). tdTom- *N/n* = 14/7, tdTom+ *N/n* = 15/7. **c** Top: Representative eEPSCs recorded at -70mV (bottom trace) and 40mV (top trace) to determine AMPAR and NMDAR current amplitudes, respectively. Bottom: AMPAR/NMDAR current ratios were similar in tdTom$^+$ and tdTom$^-$ neurons (Unpaired t-test: $t_{25} = 0.7$, $p = 0.51$). tdTom$^-$ *N/n* = 14/7, tdTom$^+$ *N/n* = 13/7). **d** Top: representative trace of a PPR recording with 100 ms ISI. PPR showed a trend towards an interaction effect, but did not reach significance (Population x ISI: $F_{1,25} = 3.9$, p = 0.06; Population: $F_{1,25} = 2.8$, p = 0.10). tdTom- *N/n*=13/7, tdTom+ *N/n*=14/7. Bar graphs show mean + s.e.m. Individual data points are neurons. Color coding of representative traces matches the bar graphs. Source data are provided as a Source Data file.



Supplementary Figure 3. Spine density analysis in mice that experienced context exposure alone. Neurons used in eEPSC recordings were filled with biocytin for post hoc spine density analysis. **a** Schematic of a layer 5 pyramidal cell with apical tuft, apical trunk and basal dendrites. **b** Spine density did not differ between tdTom$^+$ and tdTom$^-$ neurons at any dendritic segment (Mixed effects model: Population $F_{1,26} = 0.3$, $p = 0.60$; Population x Segment $F_{2,46} = 0.3$, $p = 0.77$). Tuft: tdTom+, N/n=11/7, tdTom-, N/n=14/6; Trunk: tdTom+, N/n=14/7, tdTom-, N/n=11/7; Basal: tdTom+, N/n=14/7, tdTom-, N/n=13/7. Bar graphs show mean + s.e.m. Individual data points are neurons. Source data are provided as a Source Data file.



Supplementary Figure 4. Spontaneous excitatory transmission is unaltered in mice that underwent context exposure alone. PL neurons were tagged after exposure to the conditioning context in absence of a foot shock (No US) and mEPSCs were recorded from neighboring tdTom$^+$ and tdTom$^-$ layer 5 PNs 28-31 days later (remote timepoint). **a** Representative traces of mEPSC recordings from tdTom$^-$ (grey) and tdTom$^+$ (green) PNs. **b** mEPSC amplitude did not differ between populations (Mann-Whitney U test: $U = 206$, $p = 0.41$; tdTom$^-$ N/n = 22/7, tdTom$^+$ N/n = 22/7). **c** Cumulative probability plot of mEPSC amplitudes showed a leftward shift for tdTom$^+$ neurons (Kolmogorov Smirnov test: D = 0.052, $p = 0.006$. **d** Bar graph of mean mEPSC frequency in tdTom$^-$ and tdTom$^+$ neurons (Mann-Whitney U test: $U = 187$, $p = 0.20$). tdTom$^-$ N/n = 22/7, tdTom$^+$ N/n = 22/7. **e** Cumulative probability plot of mEPSC frequencies showed a leftward shift for tdTom$^+$ neurons (Kolmogorov Smirnov test: D = 0.076, $p < 0.001$). Bar graphs show mean + s.e.m. Individual data points are neurons. Source data are provided as a Source Data file.

Excitability parameters	1US recent						1US remote					
	tdTom-			tdTom+		*p* value	tdTom-			tdTom+		*p* value
Membrane capacitance (pF)	114.32	±	7.12	115.01	± 5.09	0.94	98.78	±	7.22	111.62	± 6.38	0.20
RMP (mV)	-64.92	±	0.83	-64.84	± 0.91	0.95	-66.33	±	1.47	-66.68	± 0.72	0.84
Input resistance (MΩ)	112.36	±	12.16	105.41	± 10.03	0.66	125.65	±	13.88	128.24	± 10.48	0.88
τ_m (ms)	22.33	±	3.29	20.55	± 2.04	0.87	22.77	±	2.41	25.14	± 2.05	0.46
Sag ratio (%)	13.35	±	1.89	11.48	± 1.42	0.44	9.05	±	1.96	7.14	± 1.72	0.49
Current threshold (pA)	134.12	±	10.54	141.18	± 11.75	0.66	129.23	±	14.43	125.00	± 10.78	0.82
Ratio ISI_1/ISI_n	0.60	±	0.06	0.67	± 0.05	0.38	0.52	±	0.06	0.56	± 0.04	0.58
50% AP width (ms)	0.73	±	0.02	0.72	± 0.02	0.94	0.70	±	0.02	0.71	± 0.02	0.79
dv/dt_{max} (mV/ms)	457.65	±	19.56	446.92	± 18.97	0.70	476.52	±	25.17	476.97	± 20.64	0.99
dv/dt_{min} (mV/ms)	-100.98	±	3.30	-99.53	± 3.11	0.75	-105.23	±	4.38	-103.19	± 3.44	0.72
Voltage threshold (mV)	-38.61	±	0.78	-37.75	± 0.84	0.66	-38.99	±	0.64	-39.48	± 0.78	0.63
fAHP (mV)	-6.03	±	0.41	-7.18	± 0.69	0.16	-5.38	±	0.52	-6.16	± 0.58	0.33
mAHP (mV)	-13.57	±	0.33	-14.17	± 0.59	0.38	-13.83	±	0.39	-14.71	± 0.44	0.15
AP amplitude (mV)	88.29	±	1.53	86.32	± 1.49	0.36	86.88	±	1.79	88.12	± 1.27	0.58

Supplementary Table 1. Excitability parameters of 1US CTC tagged PL PNs in comparison to non-tagged PNs at the recent and remote memory timepoint. Values are mean ± s.e.m. P-values were determined by Student *t* test or Mann Witney *U*-test depending on normality of the data distribution. RMP = resting membrane potential, τ_m = membrane time constant, AP = action potential, fAHP = fast after-hyperpolarization, mAHP = medium after-hyperpolarization, dv/dt_{max} = peak depolarization velocity, dv/dt_{min} = min peak repolarization velocity, ISI = interspike interval.

Excitability parameters	3US recent						3US remote					
	tdTom-			tdTom+		*p* value	tdTom-			tdTom+		*p* value
Membrane capacitance (pF)	95.71	±	5.14	89.95	± 5.57	0.35	88.75	±	5.91	92.24	± 4.93	0.65
RMP (mV)	-68.64	±	1.80	-69.92	± 1.49	0.60	-67.52	±	0.91	-67.69	± 0.53	0.43
Input resistance (MΩ)	137.00	±	11.77	149.85	± 14.29	0.49	96.78	±	8.33	96.14	± 6.15	0.95
τ_m (ms)	30.18	±	4.14	27.78	± 3.57	0.66	16.75	±	1.28	19.79	± 1.83	0.31
Sag ratio (%)	13.29	±	1.44	10.50	± 1.96	0.26	16.49	±	2.31	16.84	± 1.48	0.90
Current threshold (pA)	129.64	±	8.13	136.00	± 13.37	0.69	128.21	±	8.59	151.07	± 14.56	0.33
Ratio ISI_1/ISI_n	0.52	±	0.03	0.52	± 0.04	0.95	0.54	±	0.05	0.56	± 0.03	0.67
50% AP width (ms)	1.10	±	0.04	1.15	± 0.03	0.25	1.06	±	0.03	1.05	± 0.03	0.81
dv/dt_{max} (mV/ms)	305.69	±	14.25	274.77	± 16.23	0.16	332.18	±	20.16	328.36	± 25.60	0.77
dv/dt_{min} (mV/ms)	-82.58	±	2.63	-77.89	± 2.22	0.18	-88.34	±	2.96	-88.38	± 2.78	0.99
Voltage threshold (mV)	-39.57	±	0.68	-37.95	± 0.68	0.10	-42.40	±	0.67	-41.45	± 0.58	0.29
fAHP (mV)	-4.83	±	0.55	-5.16	± 0.51	0.67	-3.91	±	0.46	-4.51	± 0.59	0.42
mAHP (mV)	-13.32	±	0.47	-13.92	± 0.57	0.42	-12.37	±	0.54	-13.01	± 0.44	0.37
AP amplitude (mV)	85.35	±	1.70	80.53	± 2.09	0.08	86.91	±	2.17	84.44	± 3.05	0.51

Supplementary Table 2. Excitability parameters of 3US CTC tagged PL PNs in comparison to non-tagged PNs at the recent and remote memory timepoint. Values are mean ± s.e.m. P-values were determined by Student *t* test or Mann Witney *U*-test depending on normality of the data distribution. RMP = resting membrane potential, τ_m = membrane time constant, AP = action potential, fAHP = fast after-hyperpolarization, mAHP = medium after-hyperpolarization, dv/dt_{max} = peak depolarization velocity, dv/dt_{min} = min peak repolarization velocity, ISI = interspike interval.

Figure	Statistical test	Sample size
1c	One-way ANOVA $F_{2,9}$=43.69, p<0.001; Post-hoc Bonferroni tests: HC -4TM *vs*. HC +4TM p=0.007, HC -4TM *vs*. CTC +4TM p<0.001, HC +4TM *vs*. CFC +4TM p=0.002	HC- n=4 HC+4TM n=4 CFC+4TM n=4
1e	Unpaired t-test: t_{12}=2.8, p=0.015	mCherry n=9 hM4Di-mCherry n=10
1f	Unpaired t-test: t_{16}=0.6, p=0.563	mCherry n=10 hM4Di-mCherry n=8
2c	Resting membrane potential, Unpaired t-test: t_{32}=0.062, p=0.95 Input resistance, Unpaired t-test: t_{32}=0.4, p=0.66	tdTom- *N/n*=17/4 tdTom+ *N/n*=17/4
2d	Mixed-effects model: Population x Current: $F_{24, 757}$=0.5, p=0.98; Population: $F_{1,32}$=0.2, p=0.66	tdTom- *N/n*=17/4 tdTom+ *N/n*=17/4
2e	Resting membrane potential, Unpaired t-test: $t_{17.55}$=0.2, p=0.84 Input resistance, Unpaired t-test: t_{25}=0.15, p=0.88	tdTom- *N/n*=13/4 tdTom+ *N/n*=14/4
2f	Population x Current: $F_{24,595}$=0.2, p=0.99; Population: $F_{1,25}$=0.01, p=0.92	tdTom- *N/n*=13/4 tdTom+ *N/n*=14/4
2g	Resting membrane, potential Unpaired t-test: $t_{20.9}$=1.5, p=0.16; Input resistance, Unpaired t-test: t_{27}=0.7, p=0.50	tdTom- *N/n*=14/3; tdTom+ *N/n*=15/3
2h	Two-way RM ANOVA: Population x Current: $F_{24, 648}$=0.3, p=0.99; Population: $F_{1, 27}$=0.3, p=0.57	tdTom- *N/n*=14/3; tdTom+ *N/n*=15/3
2i	Resting membrane potential, Mann-Whitney U-test: U=80.5, p=0.43; Input resistance, Unpaired t-test: t_{26}=0.062, p=0.95	tdTom- *N/n*=14/4; tdTom+ *N/n*=14/4
2j	Two-way RM ANOVA: Population x Current: $F_{24, 325}$=0.51, p=0.97 Population effect $F_{1,325}$=82.7, p<0.001	tdTom- *N/n*=14/4; tdTom+ *N/n*=14/4
3c	Mixed-effects model: Population x Stimulation: $F_{7,214}$=0.2, p=0.98 Population: $F_{1,31}$=0.26, p=0.62	tdTom- *N/n*=15/7 tdTom+ *N/n*=18/7
3d	Unpaired t-test: t_{30}=1.2, p=0.23;	tdTom- *N/n*=14/7 tdTom+ *N/n*=18/7
3e	Two-way RM ANOVA: Population x ISI: $F_{1,27}$=0.6, p=0.42 Population: $F_{1,27}$ =0.1, p=0.77	tdTom- *N/n*=13/7 tdTom+ *N/n*=16/7
3f	Two-way RM ANOVA: Population x Stimulation: $F_{1,2.4}$=2.5, p=0.08. Population effect: $F_{1,4}$=5.3, p=0.027. Post-hoc Bonferroni test: 12μA tdTom+ vs. tdTom- p=0.028; 14μA tdTom+ vs. tdTom- p=0.007	tdTom- *N/n*=18/9 tdTom+ *N/n*=20/9
3g	Unpaired t-test: t_{34}=0.2, p=0.85	tdTom- *N/n*=18/9 tdTom+ *N/n*=18/9
3h	Two-way RM ANOVA: Population x ISI: $F_{1,38}$<0.001, p=0.99 Population $F_{1,38}$=5.1, p=0.031.	tdTom- *N/n*=20/9 tdTom+ *N/n*=20/9

3i	Mixed-effects model: Population x Stimulation: $F_{7,188}$=0.6, p=0.76 Population $F_{1,27}$=1.0, p=0.33	tdTom- *N/n*=15/7 tdTom+ *N/n*=14/7
3j	Unpaired t-test: t_{20}=0.3, p=0.74	tdTom- *N/n*=11/6 tdTom+ *N/n*=11/6
3k	Two-way RM ANOVA: Population x ISI = $F_{1,22}$=0.6, p=0.42 Population $F_{1,22}$ = 1.3, p = 0.27	tdTom- *N/n*=12/6 tdTom+ *N/n*=12/6
3l	Mixed-effects model: Population x Stimulation: $F_{7,241}$=2.5, p=0.016 Population effect $F_{1,35}$=4.6, p=0.038. Post-hoc Bonferroni test: 14 µA tdTom+ vs. tdTom- p=0.006;	tdTom- *N/n*=17/8 tdTom+ *N/n*=20/8
3m	Unpaired t-test: t_{29}=0.91, p=0.37;	tdTom- *N/n*=15/7 tdTom+ *N/n*=16/7
3n	Two-way RM ANOVA: Population x ISI $F_{1,27}$=0.3, p=0.53 Population $F_{1,27}$=6.7, p=0.016	tdTom- *N/n*=14/7 tdTom+ *N/n*=15/7
4c	Mixed effects model: Population x Segment: $F_{2,35}$=1.3, p=0.29 Population: $F_{1,29}$=0.6, p=0.45	Tuft: tdTom+, N/n=13/6, tdTom-, N/n=11/7 Trunk: tdTom+, N/n=11/7, tdTom-, N/n=8/6 Basal: tdTom+, N/n=17/7, tdTom-, N/n=10/6
4d	Mixed effects model: Population x Segment: $F_{2,56}$=1.2, p=0.32 Population: $F_{1,30}$=6.4, p=0.017 Post-hoc Bonferroni test: Tuft: p=0.27 Trunk: p=0.031 Basal: p=0.59	Tuft: tdTom+, N/n=18/8, tdTom-, N/n=13/7 Trunk: tdTom+, N/n=18/8, tdTom-, N/n=12/7 Basal: tdTom+, N/n=17/8, tdTom-, N/n=14/7
4e	Mixed effects model: Population x Segment: $F_{2,31}$=0.5, p=0.90 Population: $F_{1,29}$=0.1, p=0.75	Tuft: tdTom+, N/n=9/5, tdTom-, N/n=7/4 Trunk: tdTom+, N/n=11/5, tdTom-, N/n=9/5 Basal: tdTom+, N/n=10/5, tdTom-, N/n=9/5
4f	Mixed effects model: Population x Segment: $F_{2,51}$=0.1, p=0.32 Population: $F_{1,29}$=4.7, p=0.039 Post-hoc Bonferroni test: Tuft: p=0.25 Trunk: *p>0.99* Basal: p=0.47	Tuft: tdTom+, N/n=18/6, tdTom-, N/n=13/7 Trunk: tdTom+, N/n=18/6, tdTom-, N/n=13/7 Basal: tdTom+, N/n=15/5, tdTom-, N/n=9/7

5b	Mann-Whitney U test: U=510, p=0.06	tdTom- N/n=37/15, tdTom+ N/n=37/15
5c	Kolmogorov Smirnov test: D=0.05, p<0.001	tdTom- N/n=3663/15, tdTom+ N/n=3663/15
5d	Mann-Whitney U test: U=522, p=0.039	tdTom- N/n=37/15, tdTom+ N/n=37/15
5e	Kolmogorov Smirnov test: D=0.06, p<0.001	tdTom- N/n=3662/15, tdTom+ N/n=3662/15
5g	Mann-Whitney U test: U=199, p=0.46	tdTom- N/n=20/8, tdTom+ N/n=23/8
5h	Kolmogorov Smirnov test: D=0.03, p=0.20	tdTom- N/n=1980/8, tdTom+ N/n=2277/8
5i	Mann-Whitney U test: U=204, p=0.54	tdTom- N/n=20/8, tdTom+ N/n=23/8
5j	Kolmogorov Smirnov test: D=0.03, p=0.24	tdTom- N/n=1980/8, tdTom+ N/n=2276/8
6c	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=1.28, p=0.26	tdTom-: N_{spines} = 2809, $n_{dendrite}$ = 41, n_{cell} = 13, $n_{section}$ = 12, tdTom+: N_{spines} = 5117, $n_{dendrite}$ = 63, n_{cell} = 19 , $n_{section}$ = 13
6d	Predictor model: b=-0.14, t=-2.05, p=0.040	tdTom-: N_{spines} = 2809, $n_{dendrite}$ = 41, n_{cell} = 13, $n_{section}$ = 12, tdTom+: N_{spines} = 5117, $n_{dendrite}$ = 63, n_{cell} = 19 , $n_{section}$ = 13
6e	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=0.10, p=0.75	tdTom-: N_{spines} = 2809, $n_{dendrite}$ = 41, n_{cell} = 13, $n_{section}$ = 12, tdTom+: N_{spines} = 5117, $n_{dendrite}$ = 63, n_{cell} = 19 , $n_{section}$ = 13
6f	Predictor model: b=-0.12, t=-2.13, p=0.033	tdTom-: N_{spines} = 4117, $n_{dendrite}$ = 61, n_{cell} = 15, $n_{section}$ = 13, tdTom+: N_{spines} = 4572, $n_{dendrite}$ = 60, n_{cell} = 15, $n_{section}$ = 13
6g	Predictor model: b=-0.19, t=-2.78, p=0.006	tdTom-: N_{spines} = 4117, $n_{dendrite}$ = 61, n_{cell} = 15, $n_{section}$ = 13, tdTom+: N_{spines} = 4572, $n_{dendrite}$ = 60, n_{cell} = 15, $n_{section}$ = 13
6h	Chi-square test random intercept vs. fixed intercept model: $\chi^2(1)$=0.59, p=0.44	tdTom-: N_{spines} = 4117, $n_{dendrite}$ = 61, n_{cell} = 15, $n_{section}$ = 13, tdTom+: N_{spines} = 4572,

		$n_{dendrite}$ = 60, n_{cell} = 15, $n_{section}$ = 13
6i	Predictor model: b=0.25, t=4.14, p<0.001	tdTom-: $N_{dendrites}$ = 41, $n_{section}$ = 12, tdTom+: $N_{dendrites}$ = 62, $n_{section}$ = 13
6j	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=3.50, p=0.062 Predictor model: b=0.26, t=1.94, p=0.053	tdTom-: $N_{dendrites}$ = 41, n_{cell} = 13, tdTom+: $N_{dendrites}$ = 62, n_{cell} = 19
6k	Predictor model: b=0.46, t=2.66, p=0.008	tdTom-: $N_{dendrites}$ = 41, n_{cell} = 13, tdTom+: $N_{dendrites}$ = 62, n_{cell} = 19
6l	Chi-square test random intercept vs. fixed intercept model: $\chi^2(1)$=0.00, p=1	tdTom-: $N_{dendrites}$ = 41, n_{cell} = 13, tdTom+: $N_{dendrites}$ = 62, n_{cell} = 19
6m	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=0.92, p=0.34	tdTom-: $N_{dendrites}$ = 61, n_{cell} = 15, tdTom+: $N_{dendrites}$ = 60, n_{cell} = 15
6n	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=3.24, p=0.07 Predictor model: b=0.15, t=1.81, p=0.07	tdTom-: $N_{dendrites}$ = 61, n_{animal} = *5*, tdTom+: $N_{dendrites}$ = 60, n_{animal} = 5
6o	Chi-square test predictor vs. random intercept model: $\chi^2(1)$=2.07, p=0.15	tdTom-: $N_{dendrites}$ = 61, n_{cell} = 15, tdTom+: $N_{dendrites}$ = 60, n_{cell} = 15
6p	Chi-square test random intercept vs. fixed intercept model: $\chi^2(1)$=0.00, p=1	tdTom-: $N_{dendrites}$ = 61, n_{cell} = 15, tdTom+: $N_{dendrites}$ = 60, n_{cell} = 15

Supplementary Table 3. Extended data related to the statistical analyses presented in the figure panels of the main text. n = number of animals, N = number of neurons, unless otherwise indicated.