Pre-synaptic HCN1 channels regulate excitatory neurotransmission at select cortical synapses by altering $Ca_V3.2$ calcium channel activity

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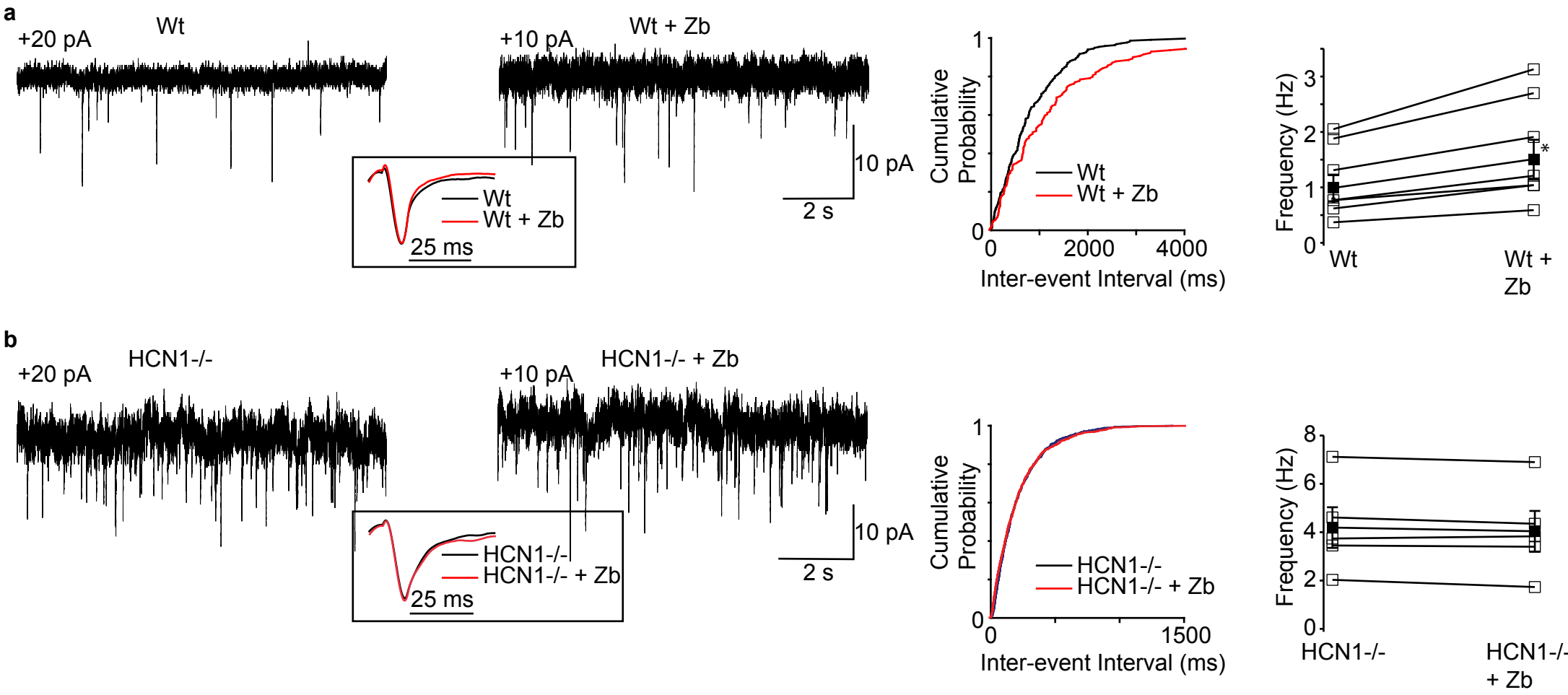
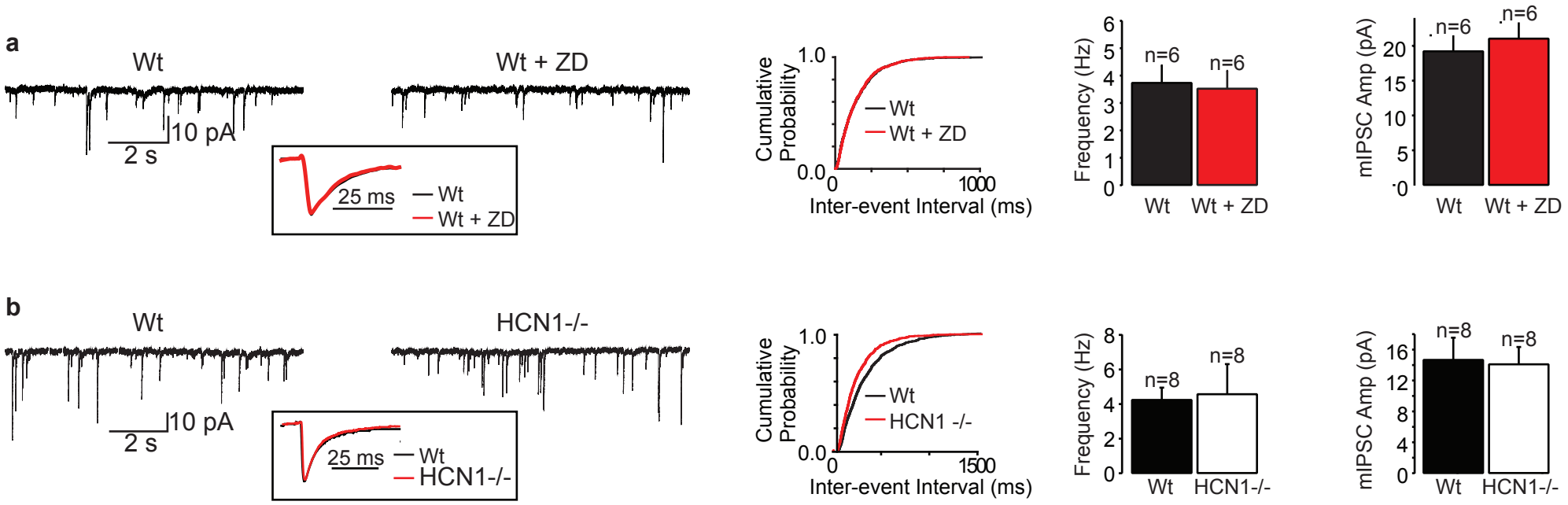
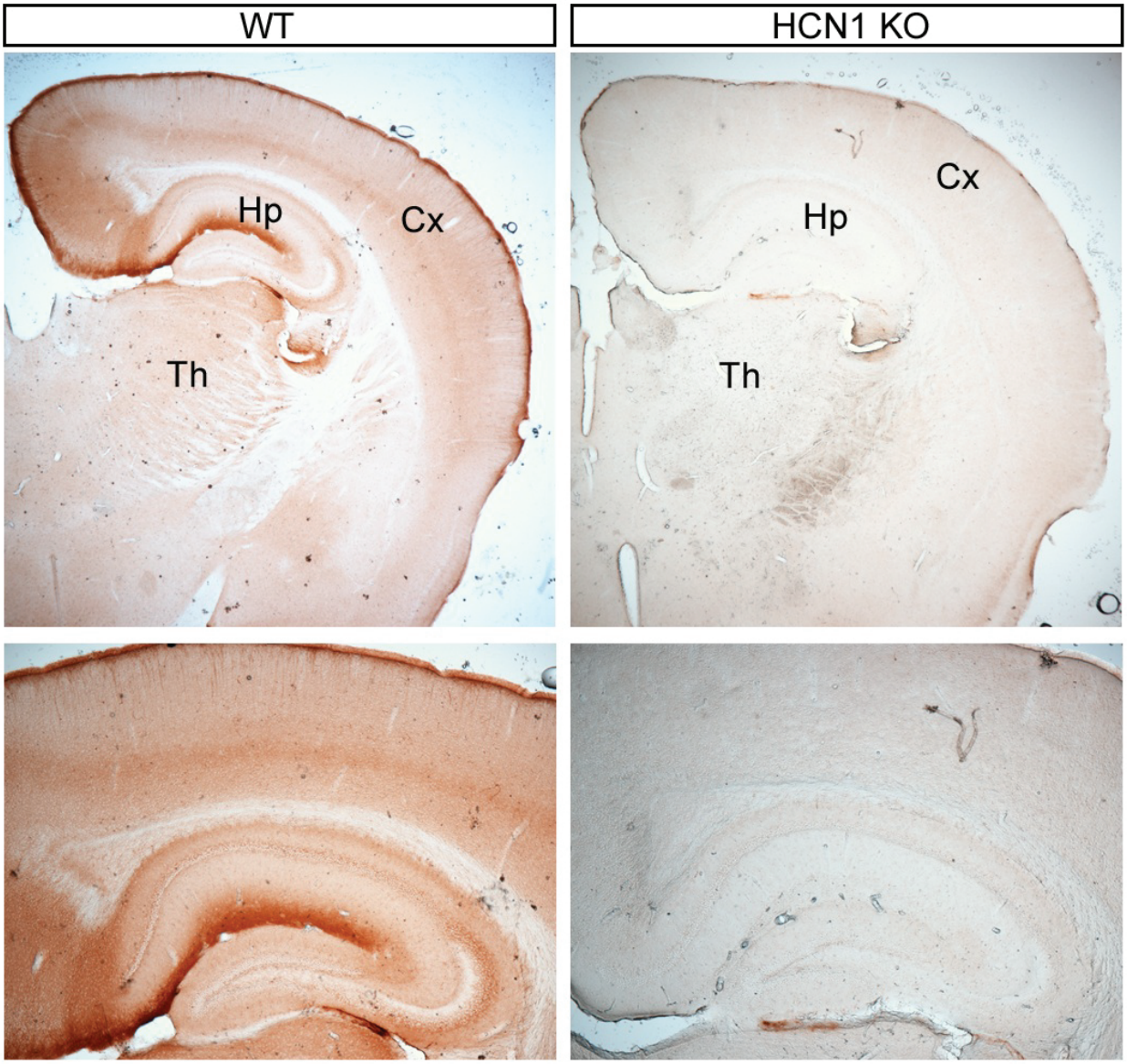


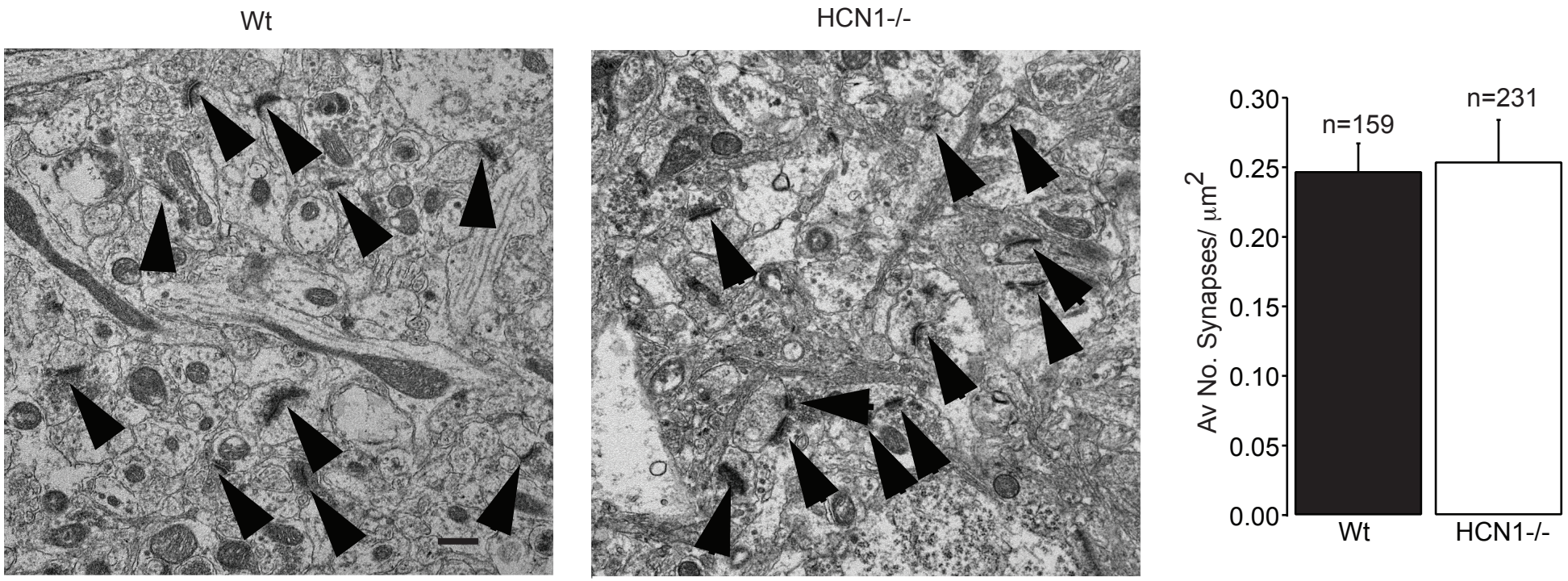
Supplementary Fig 1: Zatebradine, an HCN channel inhibitor, enhances wildtype mEPSC frequency a and b Example mEPSC recordings and cumulative probability curves from wildtype (Wt) and HCN1 null EC layer III neurons in the absence and presence of zatebradine (Zb, 10 μM) respectively. The average normalized mEPSCs before and after 15 min application of zatebradine are shown in the insets. The far right graphs show the individual frequency (open squares) as well as the mean frequency (closed squares) and standard error of mEPSCs obtained from 7 Wt neurons (**a**) and 5 HCN1 null neurons (**b**). The scale shown in **a** and **b** applies to all traces within those panels.



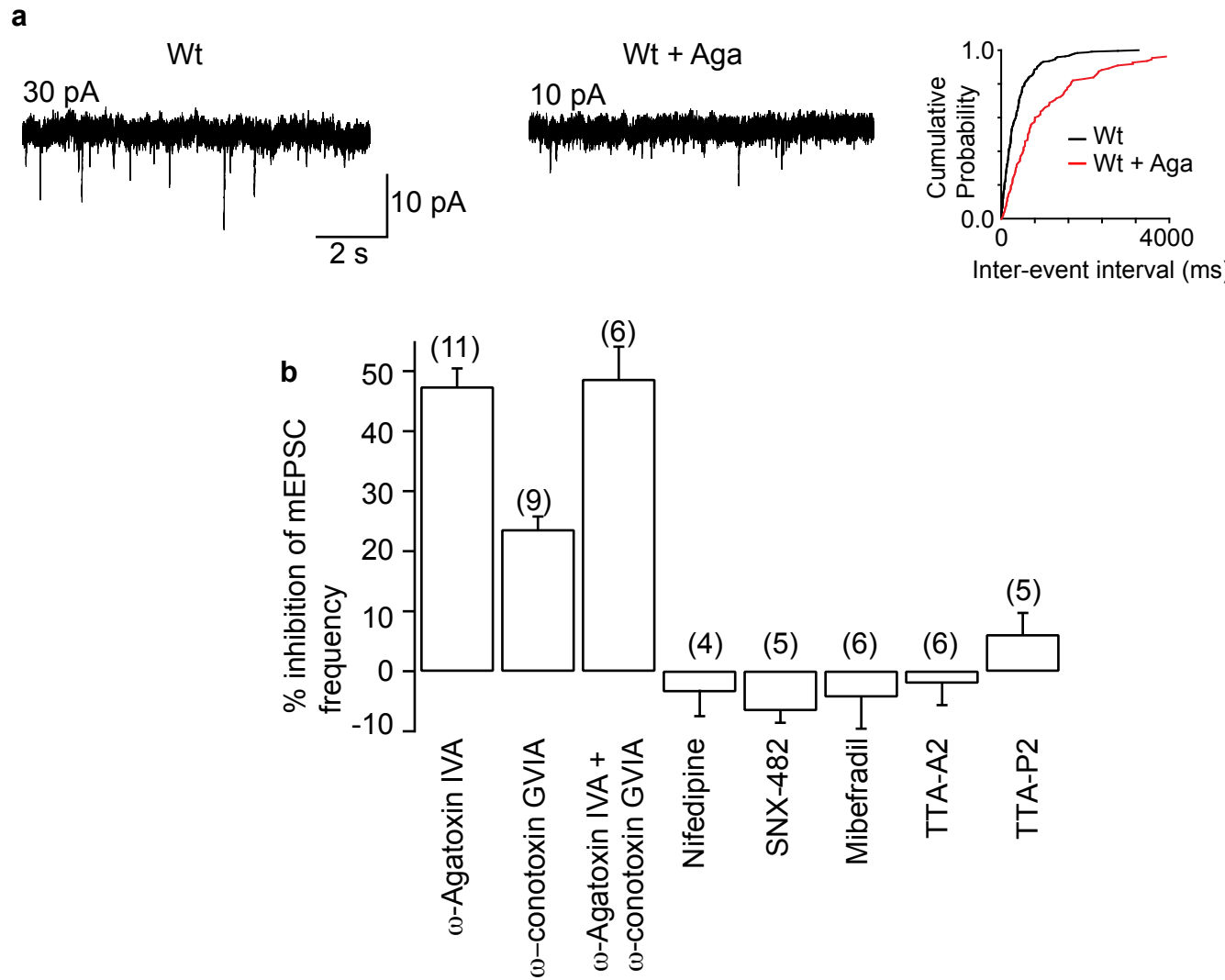
Supplementary Fig 2: mIPSC frequency unaffected by either HCN channel inhibition or deletion a Example mIPSC recordings and cumulative probability curves from wildtype (Wt) EC layer III neurons in the absence and presence of ZD7288 (ZD, 15 μM). **b** Representative traces and cumulative probability curves from wildtype and HCN1 null mice. The average normalized mIPSC recordings for **a** and **b** are presented below the traces. Both panels also show graphs comparing the mean mIPSC frequency and amplitude in wildtypes with that obtained following either pharmacological block or deletion of HCN channels. The scale bars shown in **a** and **b** apply to all traces within **a** and **b** respectively.



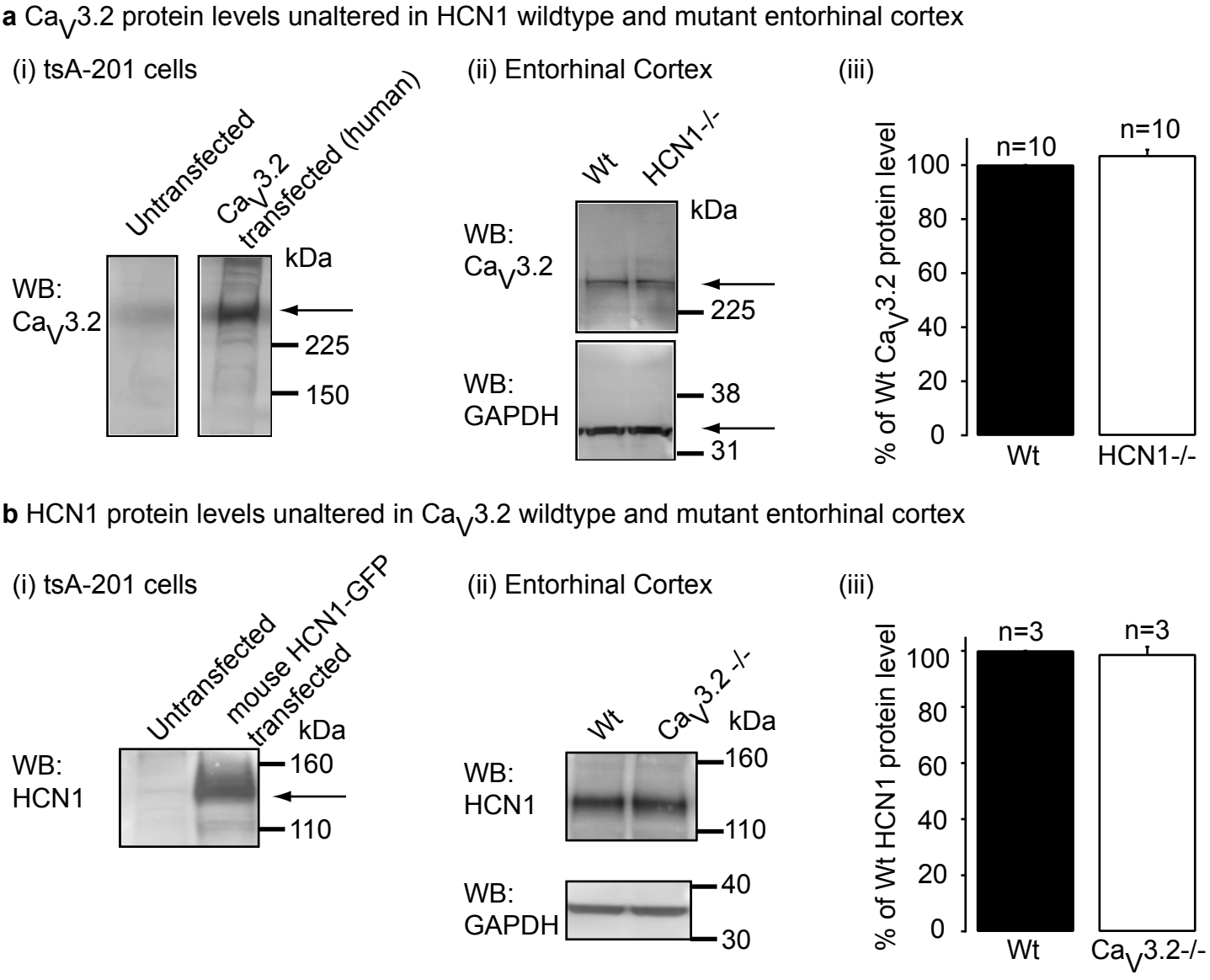
Supplementary Fig 3: **HCN1 antibody labelling in wildtype and HCN1 null sections.** Light microscopy shows clear staining with the HCN1 antibody in wildtype hippocampus (Hp) and cortex (Cx) tissue but not in the thalamus (Th), where HCN2 is predominantly expressed. In contrast, no staining was observed at the light microscopy level in HCN1 null mice.



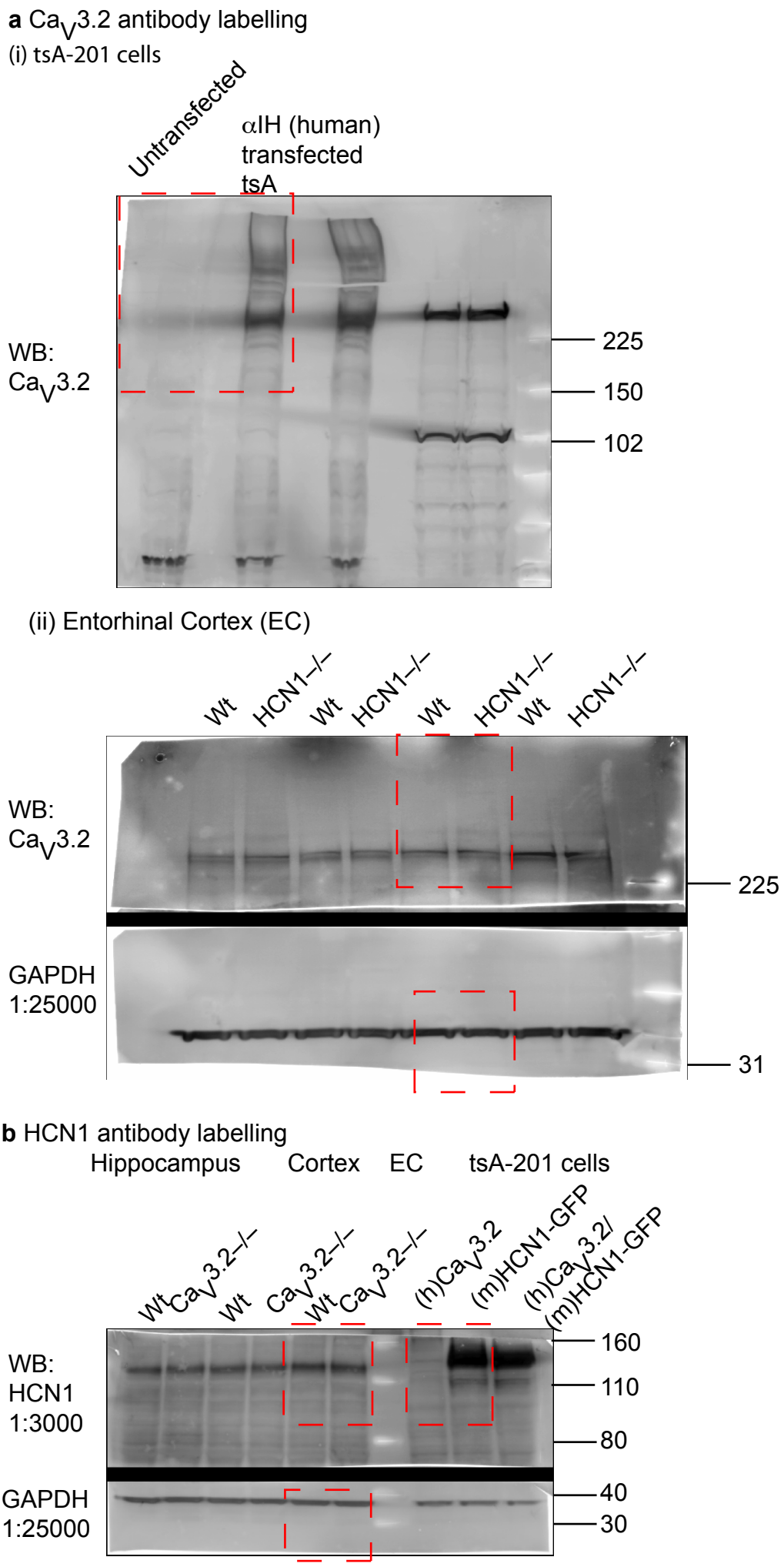
Supplementary Fig 4: **Comparison of the number of synapses in wildtype and HCN1 null EC superficial layers.** Example electron micrographs showing synaptic contacts in entorhinal cortical (EC) sections obtained from wildtype and HCN1 null mice. The arrows point to the synapses in these particular examples. The scale bar represents 500 nm and applies to both micrographs. The average no. of synaptic contacts counted from 159, 85 nm ultra-thin sections of the EC superficial layers obtained from 3 wildtypes and 231 EC ultra-thin sections from 4 HCN1 null mice is shown in the bar graph on the right.



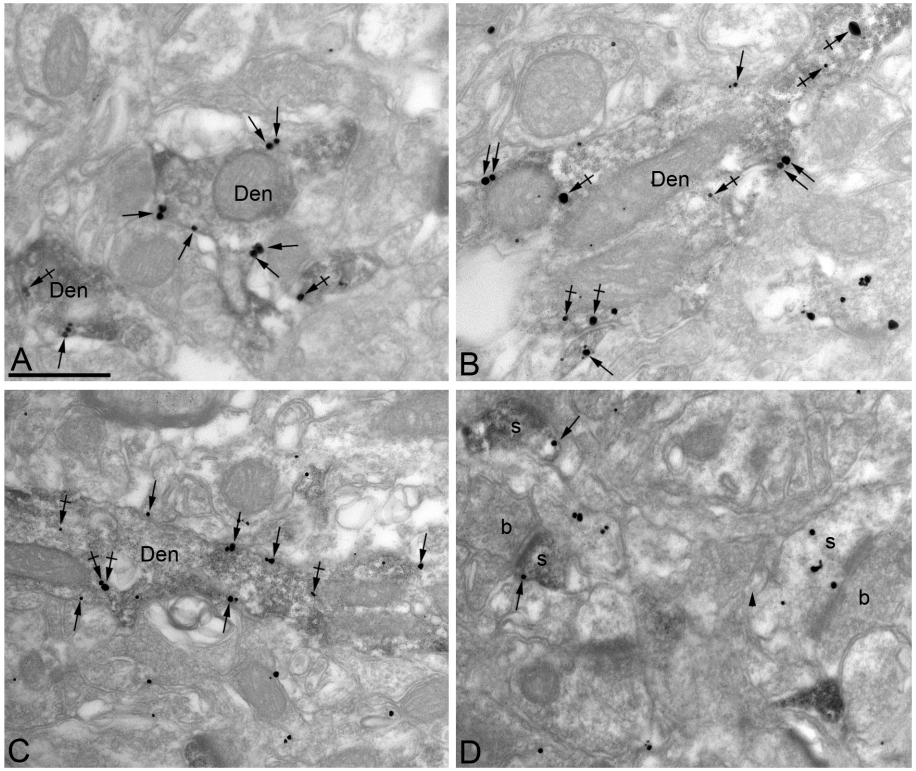
Supplementary Fig 5: Effects of Ca^{2+} channel inhibitors on wildtype mEPSCs a Example recordings of mEPSCs obtained from wildtype (Wt) EC layer III soma before and after 15 min bath application of the P/Q Ca^{2+} channel blocker ω-agatoxin IVA (Aga; 100 nM). The outward holding currents for the recordings are displayed above the traces. The cumulative probability curves for those particular examples are shown on the right. The scale shown applies to both traces. **b** Graph summarizing the effects of Ca^{2+} channel blockers on mEPSC frequency in wildtype neurons. The number of observations for each are displayed in parantheses.



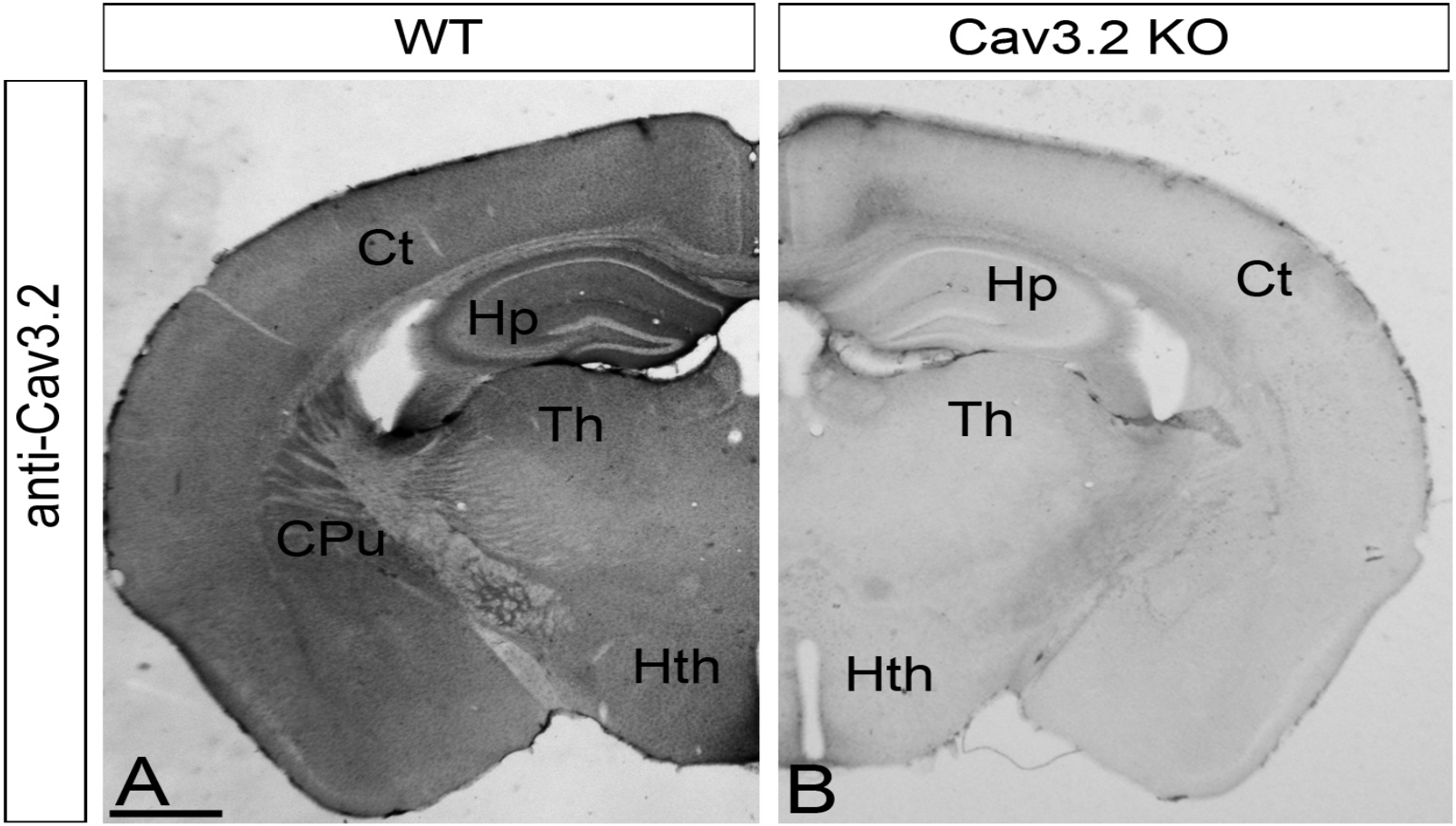
Supplementary Fig 6: **Western blots to illustrate that $Ca_V3.2$ and HCN1 protein levels are unaltered in HCN1 and $Ca_V3.2$ null EC tissue respectively. a(i) and b(i)** Example immunoblots of whole cell lysate (WCL) preparations from untransfected tsA-201 cells, human $Ca_V3.2$ transfected tsA-201 (a(i)) and mouse-HCN1-GFP transfected tsA-201 cells (b(i)). Arrow indicates $Ca_V3.2$ band (a(i)) or HCN1-GFP band (b(i)), the molecular weight of which is higher than endogenous HCN1 due to the presence of the GFP tag. **a(ii) and b(ii)** Representative immunoblots against HCN1 or $Ca_V3.2$ for WCL prepared from entorhinal cortex (EC) dissected from HCN1-/- or $Ca_V3.2$ null mice and their respective wildtype littermates. The lower parts of the same membranes were incubated with anti-GAPDH antibody, which served as a control for the amount of protein loading. 50 μg of protein were loaded per lane. a(iii) and b(iii) Quantification of the $Ca_V3.2$ and HCN1 concentrations in HCN1 null and $Ca_V3.2$ null EC respectively as a percentage of that detected in wildtype tissue. The data are obtained from either 10 ($Ca_V3.2$, a(iii)) or 3 (HCN1, b(iii)) independent preparations from different animals. The values obtained were normalised to the level of GAPDH for each preparation. The full length Western blots are illustrated in **Supplementary Fig 7**.



Supplementary Fig 7: Full length Western blots for $Ca_V3.2$ and HCN1 antibodies. a and **b** Full length Western blots for cropped versions (red boxes) shown in **Supplementary Fig 6a** and **b**. NB Polyvinylidene fluoride (PVDF) membranes were cut in these experiments and full length blots, therefore, are not always available.



Supplementary Fig 8: Co-localization of HCN1 and $Ca_V3.2$ at post-synaptic sites in the EC. (A-B) Examples showing immunoreactivity for HCN1 and $Ca_V3.2$ in the same dendritic shafts and spines, as detected using pre-embedding double-labelling methods at EM level. (A-B) The peroxidise reaction product (HCN1 immunoreactivity) is present in dendrites (Den) and dendritic spines (s) establishing excitatory synapses with axon terminals, whereas immunoparticles ($Ca_V3.2$ immunoreactivity) were located along the extrasynaptic plasma membrane (arrows) of dendrites and spines, as well as at intracellular sites (crossed arrows). (C-D) The peroxidise reaction product ($Ca_V3.2$ immunoreactivity) in dendrites (Den) and dendritic spines (s) establishing excitatory synapses with axon terminals (b), whereas immunoparticles (HCN1 immunoreactivity) were located along the extrasynaptic plasma membrane (arrows) of dendrites and spines, as well as at intracellular sites (crossed arrows). Scale bars: A-D, 0.5 µm.



Supplementary Fig 9: $Ca_V3.2$ labelling is absent in $Ca_V3.2$ null tissue. (A) Immunoreactivity for $Ca_V3.2$ in wildtype (Wt) sections at light microscopy level. Immunoreactivity was detected throughout the brain and was especially intense in the cortex (Ct) and hippocampus (Hp). (B) Immunoreactivity was absent in $Ca_V3.2$ null tissue. Cpu, caudate putamen; Th, thalamus; Hth, hypothalamus. Scale bar in (A) represents 1 mm and applies to both sections.

	HCN1-/- Soma without ZD in the pipette	Wt Soma mEPSC without ZD in the pipette	Wt Soma mEPSC with ZD in the pipette	HCN1-/- Soma with ZD in the pipette	HCN1-/- Dendrite with ZD in the pipette	Wt Dendrite with ZD in the pipette	Wt Dendrite with Cs + ZD in the internal solution	Wt Soma with Cs + ZD in the internal solution
Frequency (Hz)	5.6 ± 0.8 (n=18)	1.9 ± 0.2 (n=13)	2.0 ± 0.1 (n=141)	5.5 ± 0.3 (n=103)*	3.8 ± 0.5 (n=7)*	2.6 ± 0.4 (n=13)	2.7 ± 0.3 (n=4)	1.6 ± 0.2 (n=15)
Amplitude (pA)	6.4 ± 0.4 (n=18)	5.1 ± 1.1 (n=13)	5.4 ± 0.1 (n=141)	4.9 ± 0.1 (n=103)*	6.3 ± 0.9 (n=7)	5.6 ± 0.5 (n=13)	4.9 ± 0.2 (n=4)	4.7 ± 0.3 (n=15)
Decay time constant (ms)	9.4 ± 0.6 (n=18)	8.1 ± 0.5 (n=13)*	9.8 ± 0.3 (n=141)	9.6 ± 0.3 (n=103)	8.4 ± 0.5 (n=7)	9.2 ± 1.0 (n=13)	10.5 ± 0.7 (n=4)	9.8 ± 1.0 (n=15)
Rise time constant (ms)	1.3 ± 0.1 (n=18)	1.3 ± 0.1 (n=13)	1.4 ± 0.0 (n=141)	1.3 ± 0.0 (n=103)	1.4 ± 0.1 (n=7)	1.3 ± 0.1 (n=13)	1.3 ± 0.2 (n=4)	1.5 ± 0.1 (n=15)
Outward holding current at -70 mV(pA)	16.7 ± 3.1 (n=18)	-21.1 ± 7.2 (n=9)*	21.2 ± 2.4 (n=141)	23.9 ± 3.4 (n=103)	35.7 ± 21.4 (n=7)	13.9 ± 5.0 (n=13)	-7.5 ± 5.8 (n=4)	7.0 ± 2.6 (n=15)

Supplementary Table 1: **Wildtype and *HCN1-/-* mEPSC properties.** A comparison of mEPSC frequency, amplitudes and shapes as well as the outward holding current at -70 mV obtained from EC layer III soma of wildtype neurons with and without ZD7288 included in the pipette. The somatic and dendritic mEPSC frequency, amplitude and kinetics and outward holding current data recorded from HCN1-/- neurons is also shown and compared with that obtained from wildtype soma and dendrites when ZD7288 was incorporated in the patch pipette or when $KMeSO_4$ was replaced with $CsMeSO_4$ (Cs). Asterisks indicate significance at $p < 0.05$.

	Mibefradil (10 μM)	ω-agatoxin IVA (100 nM)	ω –conotoxin GVIA (100 nM)	SNX-482 (200 nM)	Nifedipine (2 μM)	TTA-A2 (500 nM)	TTA-P2 (1 μM)
% reduction in amplitude (ms)	-4.4±6.4% (6)	-8.1±4.8% (11)	-8.3±6.7% (9)	11.5±6.5% (4)	8.4±5.5% (4)	6.0±3.5% (6)	3.4±3.6% (5)
% reduction in rise time constant (ms)	6.8±9.3% (6)	-15.4±5.7%* (11)	-10.0±7.4% (9)	5.9±6.6% (4)	-6.6±6.6% (4)	-4.4±4.4% (6)	-13.0±7.2% (5)
% reduction in decay time constant (ms)	-7.6±5.6% (6)	1.7±4.2% (11)	-10.0±10.7% (9)	10.1±1.9%* (4)	3.9±5.9% (4)	-7.6±4.6% (6)	-15.9±7.9% (5)

Supplementary Table 2: **Wildtype mESPC properties in the presence of Ca^{2+} channel blockers**. Table to show the percentage inhibition in mEPSC amplitude, rise time and decay time constant in wildtype neurons following application of various Ca^{2+} channel inhibitors. The numbers of observations for each treatment are shown in parentheses. Asterisks indicate significance at $p < 0.05$.

	Mibefradil (10 μM)	TTA-A2 (500nM)	TTA-P2 (1 μM)	SNX-482 (200 nM)
% reduction in amplitude (ms)	11.1±6.5% (5)	-2.9±7.4% (6)	-3.4±3.2% (5)	-5.5±9.4% (7)
% reduction in rise time constant (ms)	-17.9±13.3% (5)	-9.0±3.7% (6)	-2.7±11.8% (5)	-15.5±20.4% (7)
% reduction in decay time constant (ms)	27.6±6.7% (5)	14.2±9.4% (6)	-4.4±9.1% (5)	7.6±13.9% (7)

Supplementary Table 3: **Effects of T- and R- type Ca^{2+} channel inhibitors on mEPSC kinetics in *HCN1*$^{-/-}$ neurons.** Table showing the effects of the T- (mibefradil, TTA-A2 and TTA-P2) and R- (SNX-482) type Ca^{2+} channel blockers on amplitude and time constants of *HCN1*$^{-/-}$ mEPSCs. The numbers of observations for each treatment are shown in parentheses. Significance ($p < 0.05$) is indicated by asterisks.

	$Ca_V3.2$ Wt neurons (n=5)	$Ca_V3.2$ null neurons (n=6)
Frequency (Hz)	1.6 ± 0.29	1.6 ± 0.21
Amplitude (pA)	4.8 ± 0.6	6.2 ± 0.4
Decay time constant (ms)	11.9 ± 1.2	9.8 ± 1.0
Rise time constant (ms)	1.5 ± 0.1	1.5 ± 0.1

Supplemental Table 4: **Comparison of $Ca_V3.2$ wildtype and null mEPSC properties**. The average frequency, amplitude, decay time constants and rise time constants of mEPSCs recorded from $Ca_V3.2$ wildtype (Wt) and null neurons. NB The HCN channel blocker, ZD7288 (15 μM) was included in the patch pipette to inhibit post-synaptic HCN channels.