Calcineurin is universally involved in vesicle endocytosis at neuronal and non-neuronal secretory cells

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Supplementary materials

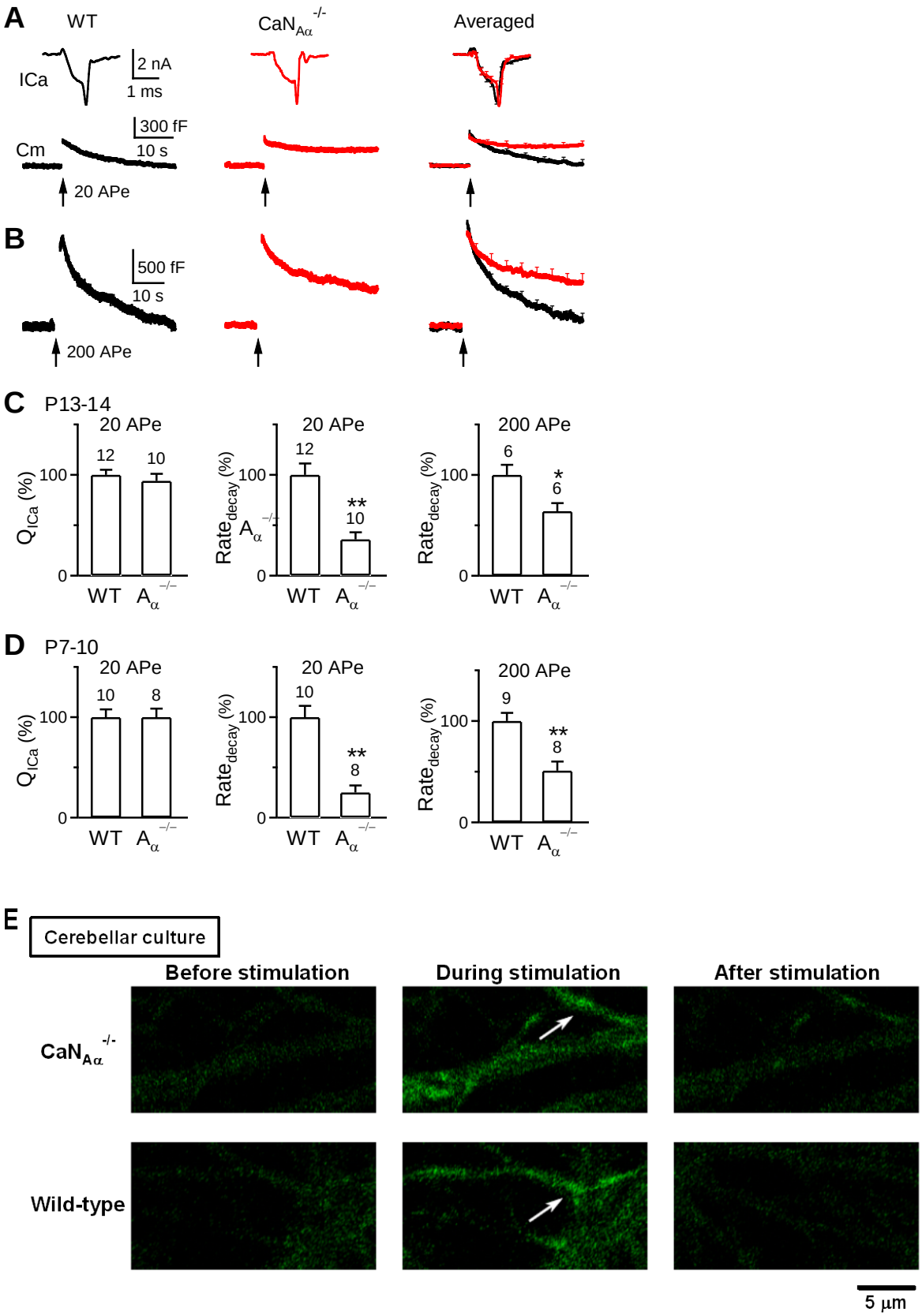
A
WT
CaN$_{A\alpha}^{-/-}$
Averaged
ICa
2 nA
1 ms
Cm
300 fF
10 s
20 APe
B
500 fF
10 s
200 APe
C
P13-14
20 APe
20 APe
200 APe
Q$_{ICa}$ (%)
Rate$_{decay}$ (%)
WT
A$_\alpha^{-/-}$
D
P7-10
E
Cerebellar culture
Before stimulation
During stimulation
After stimulation
CaN$_{A\alpha}^{-/-}$
Wild-type
5 µm

Figure S1. $CaN_{A\alpha}$ knockout inhibits endocytosis induced by action potential-equivalent trains, but does not affect calcium currents or calcium influx; Mostly related to Figure 1 (also related to Figure 3).

(A) Sampled (left and middle) and averaged (right, mean + s.e.m.) ICa and Cm in calyces induced by 20 pulses of 1 ms depolarization from -80 to +7 mV at 100 Hz (20 APe) at P13-14 wild-type (WT, black, 12 calyces) and $CaN_{A\alpha}^{-/-}$ mice (red, 10 calyces). For clarity, we only show the ICa induced by the first 1 ms depolarizing pulse. The 1 ms depolarization is called APe, because it is equivalent to an action potential in releasing the same amount of vesicles (Sun et al., 2002; Xu et al., 2008).

(B) Sampled and averaged Cm in calyces induced by 200 APe at 100 Hz in P13-14 WT (black, 6 calyces) and $CaN_{A\alpha}^{-/-}$ (red, 6 calyces) mice.

(C-D) QICa and $Rate_{decay}$ in calyces induced by 20 APe at 100 Hz and $Rate_{decay}$ induced by 200 APe at 100 Hz in WT and $CaN_{A\alpha}^{-/-}$ mice in P13-14 (C) or P7-10 calyces (D). Data were normalized to the mean of the WT group and the number of calyces was labeled above the bar. *: $p<0.05$; **: $p<0.01$.

(E) Similar calcium influx in wild-type and $CaN_{A\alpha}^{-/-}$ cerebellar culture (related to Figure 3). Sampled images showing fluo2 signal (calcium indicator) before (left), during (middle), and after (right) a 10 s train of stimulation at 20 Hz ($Train_{20Hz}$) at cerebellar cultures from $CaN_{A\alpha}^{-/-}$ mice (Upper panels) and wild-type mice (Lower panels). For fluo2 loading, we applied 5 μM fluo2-AM to the bath for 30 min, then washout. Arrows indicate bouton-like structures.

In a separate set of data, we showed that fluo2 fluorescence increase overlapped with FM dye uptake (data not shown), confirming that fluo2 signals were from axonal boutons. In a recent study, we also confirmed that the fluo2 fluorescence increase overlaps with staining of synaptophysin that labels synaptic vesicles at hippocampal synapses (Zhang et al., 2013). Thus, these fluo2 signals were from axonal boutons.

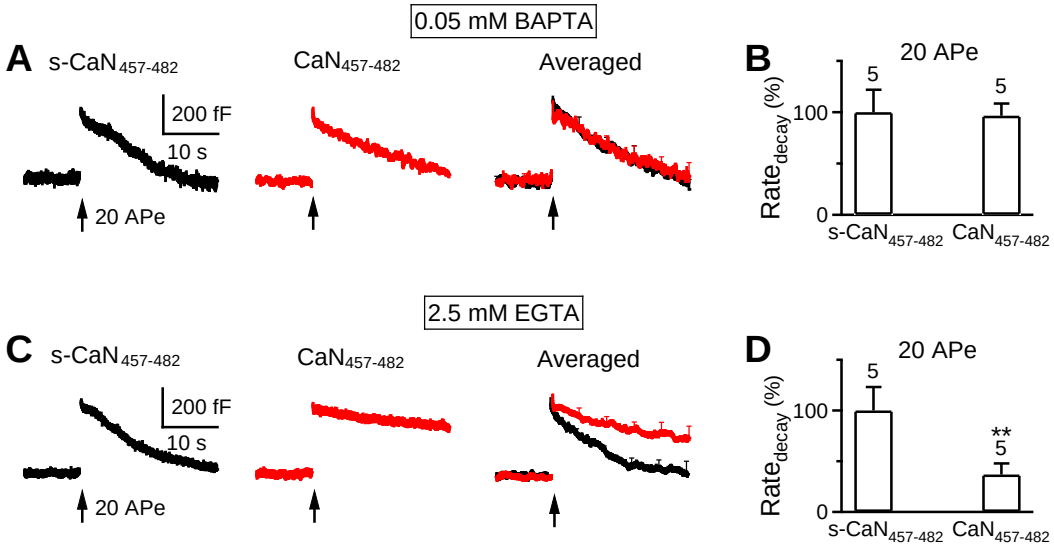

Figure S2. $CaN_{457-482}$ inhibited endocytosis induced by an APe train at a higher, but not a lower calcium buffer concentration in P13-14 rat calyces; related to Figure 2

(A) Sampled (left and middle) and averaged (right, mean + s.e.m) Cm changes induced by 20 APe at 100 Hz with a pipette solution containing 0.05 mM BAPTA and either scrambled $CaN_{457-482}$ (s-$CaN_{457-482}$, 200 μM, control, black, n = 5) or $CaN_{457-482}$ (200 μM, red, n = 5). Data were obtained from P13-14 rat calyces.

(B) $Rate_{decay}$ (mean + s.e.m.) induced by 20 APe at 100 Hz with a pipette solution containing 0.05 mM BAPTA and either scrambled $CaN_{457-482}$ (s-$CaN_{457-482}$, 200 μM, control, n = 5) or $CaN_{457-482}$ (200 μM, n = 5). Data were obtained from P13-14 rat calyces.

(C-D) Similar to panels A-B respectively, except that the 0.05 mM BAPTA was replaced with 2.5 mM EGTA. s-$CaN_{457-482}$, n = 5; $CaN_{457-482}$, n = 5. **: $p < 0.01$.

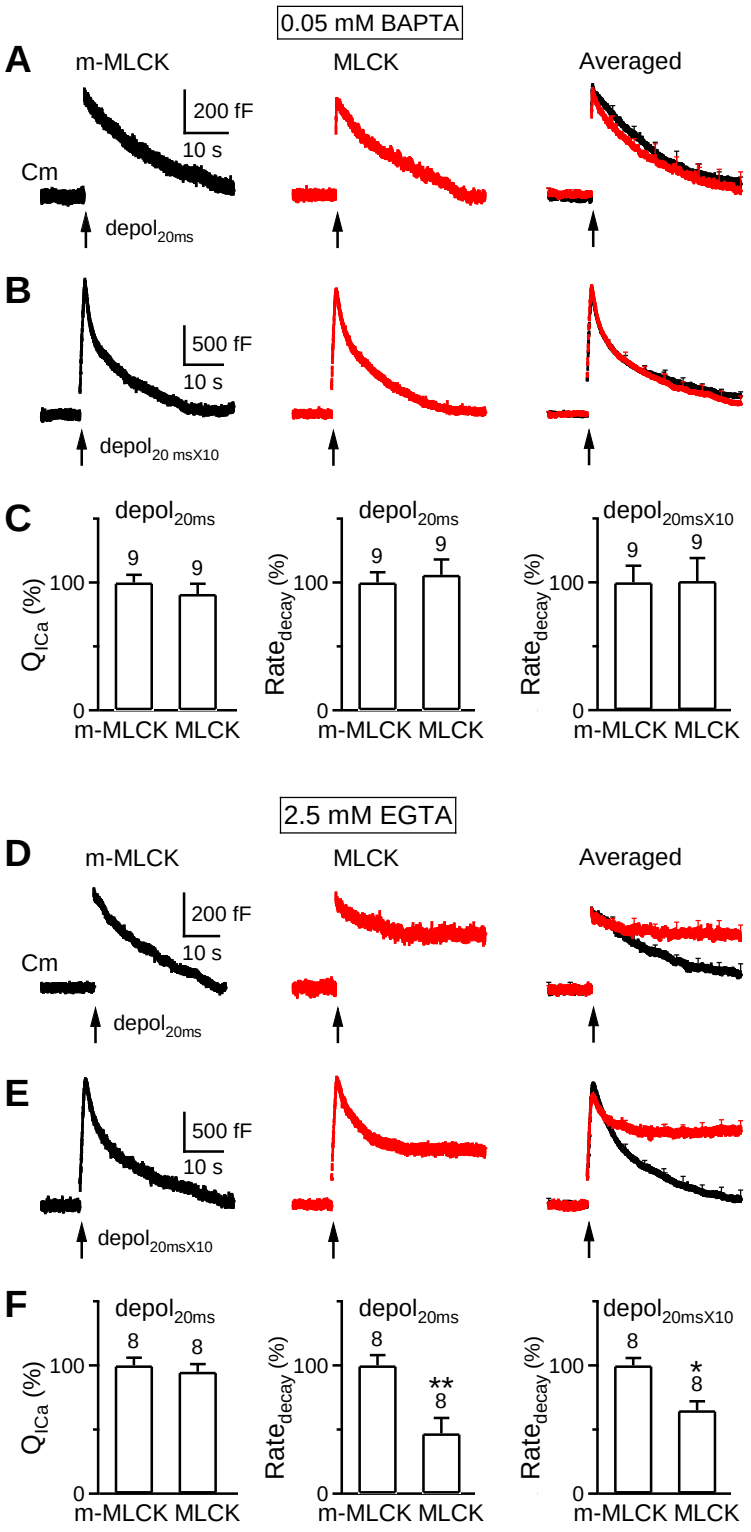
0.05 mM BAPTA
A
m-MLCK
MLCK
Averaged
200 fF
10 s
Cm
depol20ms
B
500 fF
10 s
depol20 msX10
C
depol20ms
depol20ms
depol20msX10
QICa (%)
Rate decay (%)
Rate decay (%)
100
0
9
9
9
9
9
9
m-MLCK MLCK
m-MLCK MLCK
m-MLCK MLCK
2.5 mM EGTA
D
m-MLCK
MLCK
Averaged
200 fF
10 s
Cm
depol20ms
E
500 fF
10 s
depol20msX10
F
depol20ms
depol20ms
depol20msX10
QICa (%)
Rate decay (%)
Rate decay (%)
100
0
8
8
8
8
8
8
**
*
m-MLCK MLCK
m-MLCK MLCK
m-MLCK MLCK

Figure S3. The effect of a calmodulin blocker (MLCK peptide) on endocytosis depends on the calcium buffer concentration at P13-14 rat calyces; related to Figure 2

(A) Sampled (left and middle) and averaged (right) Cm changes induced by $depol_{20ms}$ with a pipette solution containing 0.05 mM BAPTA and either mutated myosin light chain kinase peptide (m-MLCK, 20 μM, control, black, n = 9) or MLCK (20 μM, red, n = 9). Data were obtained from P13-14 rat calyces (applies to A-F).

(B) Similar to panel a, except that the stimulus was $depol_{20msX10}$ (m-MLCK, n = 9; MLCK, n = 9).

(C) QICa and $Rate_{decay}$ induced by $depol_{20ms}$ and $Rate_{decay}$ induced by $depol_{20msX10}$ in control (m-MLCK, black, n = 9) and in the presence of MLCK (red, n = 9). In both groups, the pipette contained 0.05 mM BAPTA. Data were normalized to the corresponding control group and the number of calyces is labeled above the bar. *: $p<0.05$; **: $p<0.01$.

(D-F) Similar to panels A-C, respectively, except that 0.05 mM BAPTA was replaced with 2.5 mM EGTA in the pipette solution. Panel D ($depol_{20ms}$): m-MLCK, n = 8; MLCK, n = 8. Panel E ($depol_{20msX10}$): m-MLCK, n = 8; MLCK, n = 8.

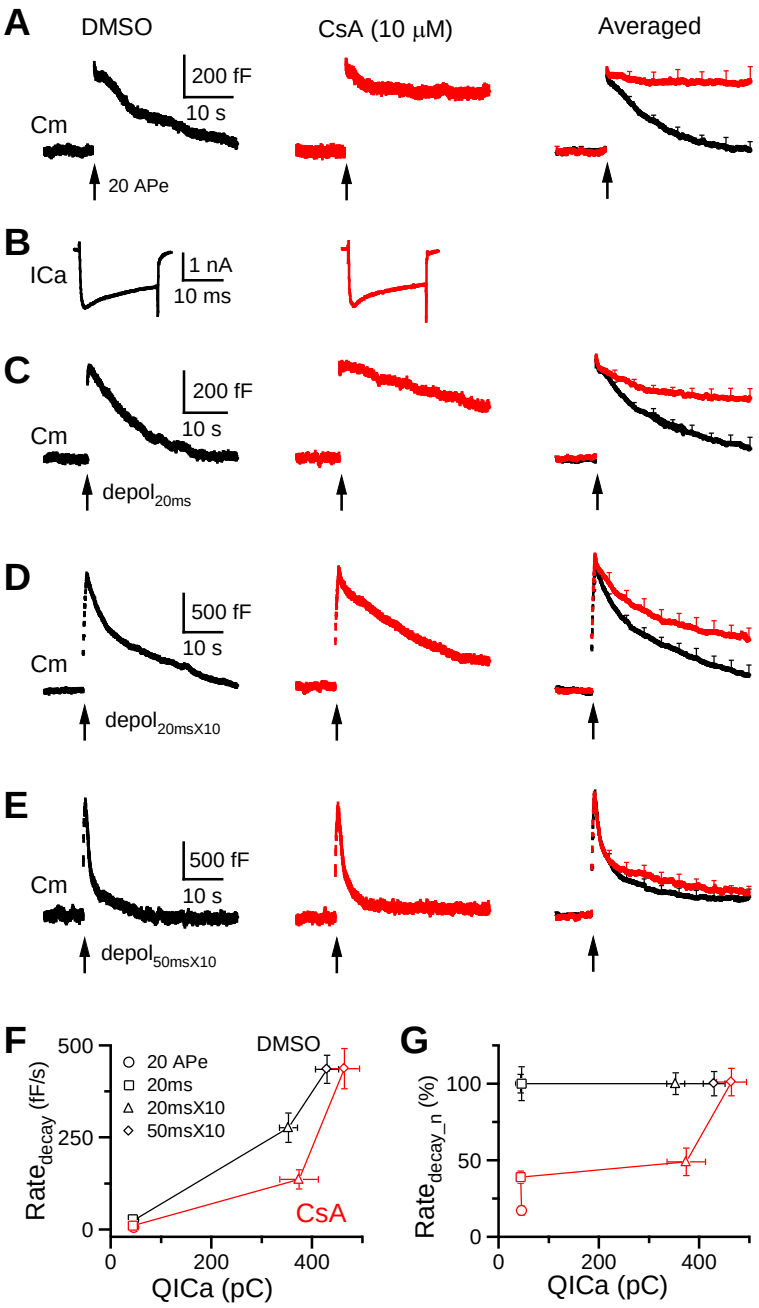

Figure S4. Calcium-dependent inhibition of endocytosis by the CaN blocker cyclosporine A at P7-10 rat calyces; related to Figure 2

(A) Sampled (left and middle) and averaged (right) Cm changes induced by 20 APe (1 ms depolarization from -80 to +7 mV) at 100 Hz with a pipette containing the control solution (containing 0.1% DMSO, control, black) or cyclosporine A (CsA, 10 µM in 0.1% DMSO, red). Averaged traces are superimposed and plotted as mean + s.e.m. (right; control, n = 10 calyces;

CsA, n = 10 calyces). The s.e.m. is plotted every 5 s for better view of the data. Data were obtained from P7-10 rat calyces (applies to panels A-G).

(B) Sampled ICa induced by $depol_{20ms}$ in control (black) and in the presence of CsA (red).

(C-E) Similar to panel A, except that the stimulus was $depol_{20ms}$ (C; control, n = 12; CsA, n = 12), $depol_{20msx10}$ (D; control, n = 11; CsA, n = 11) or $depol_{50msx10}$ (E; control, n = 11; CsA, n = 11).

(F) $Rate_{decay}$ plotted versus QICa induced by 20 APe (circles), $depol_{20ms}$ (squares), $depol_{20msX10}$ (triangles) and $depol_{50msX10}$ (diamonds) in control (black) and in the presence of CsA (red). Data are plotted as mean ± s.e.m. in both x and y axis. The number of calyces tested is indicated in panels A-E.

(G) Same as panel F, except that for each stimulus, the data are normalized to the mean $Rate_{decay}$ of the corresponding control group, so that the mean of each control group is 100% (y axis: $Rate_{decay\_n}$: normalized $Rate_{decay}$). This plot shows clearly that as QICa increased, the inhibitory effect of CsA is diminished.

Conclusion: The decrease of $Rate_{decay\_n}$ caused by CsA depended on QICa. The decrease was significant at small QICa, but negligible at very large QICa at P7-10 calyces.

Reference List

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Zhang, Z., Wang, D., Sun, T., Xu, J., Chiang, H.C., Shin, W., and Wu, L.G. (2013). The SNARE proteins SNAP25 and synaptobrevin are involved in endocytosis at hippocampal synapses. J. Neurosci. *33*, 9169-9175.