Dhara et al., http://www.jcb.org/cgi/content/full/jcb.201311085/DC1

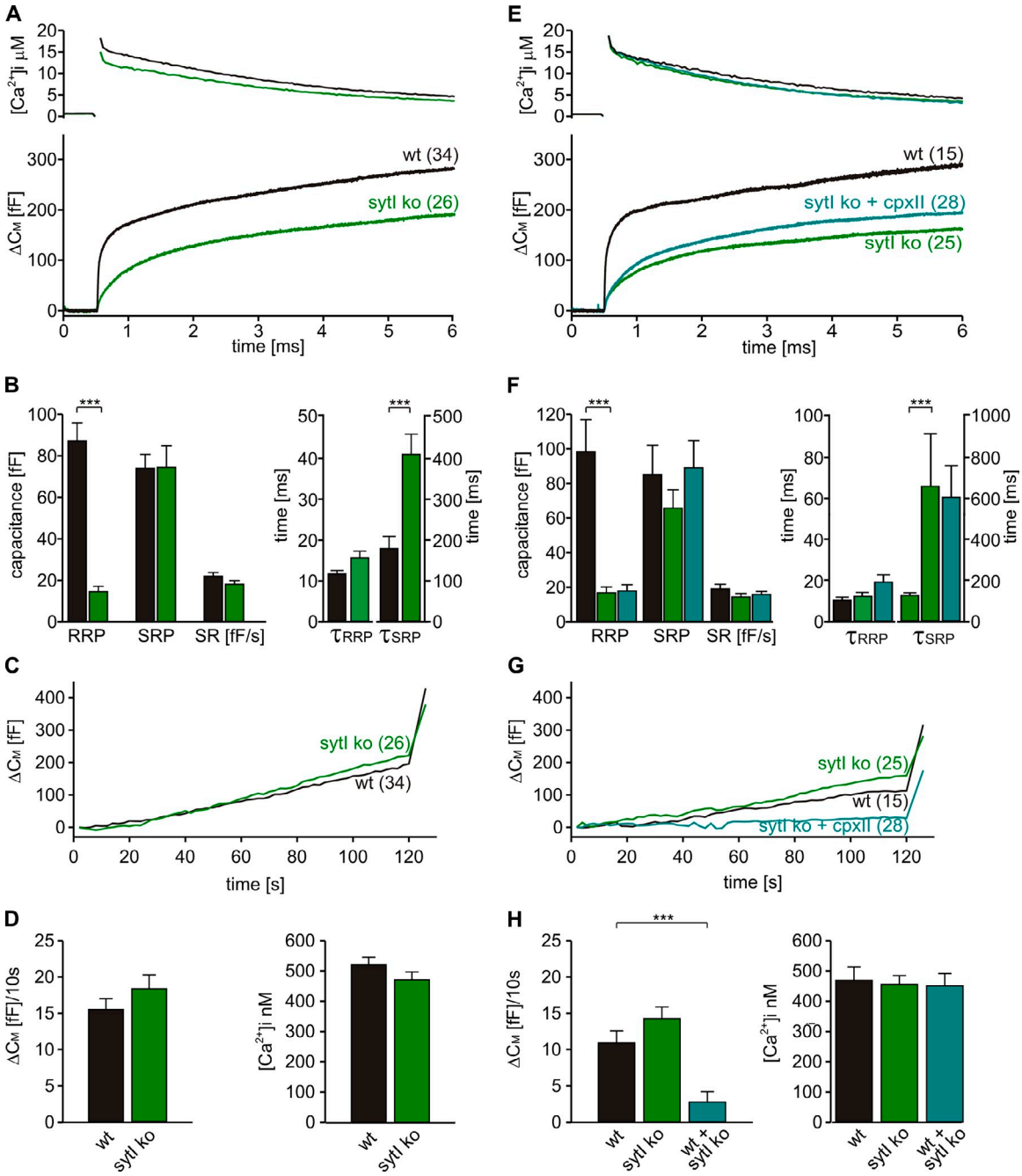

Figure S1. **CpxII clamps tonic secretion in SytI ko cells like in wt cells.** (A) Mean flash-induced $[Ca^{2+}]i$ levels (top) and the corresponding capacitance responses (bottom) of wt (n = 34) and SytI ko cells (n = 26). Flash, t = 0.5 s. (B) Loss of SytI specifically reduces the RRP component of the EB and slows the time constant of the SRP (τ_{SRP}), whereas the SRP, the sustained release phase, and the time constant of the RRP (τ_{RRP}) remain unchanged (wt, black bars; SytI ko, green bars). ***, P < 0.001, Student's t test. (C) The magnitude of tonic exocytosis in SytI ko cells is similar to that of wt cells. (D) The mean rate of tonic secretion (left) and the preflash [Ca]i values (right) for the responses shown in C are nearly identical. (E) Averaged flash-induced $[Ca^{2+}]i$ levels (top) and the corresponding capacitance responses (bottom) of wt cells (n = 15), SytI ko cells (n = 28), and SytI ko cells expressing CpxII (n = 28). Flash, t = 0.5 s. (F) Overexpression of CpxII in SytI ko cells fails to enhance the EB and leaves the kinetic properties of the SytI ko response unchanged (wt, black bars; SytI ko, green bars; and SytI ko + CpxII, cyan bars). (G) CpxII clamps tonic secretion in SytI ko cells with similar efficiency as in wt cells. (H) The mean rate of tonic secretion (left) measured at similar preflash [Ca]i (right) is significantly reduced in SytI ko cells expressing CpxII. ***, P < 0.001, one-way ANOVA for F and H. Error bars indicate means ± SEM.

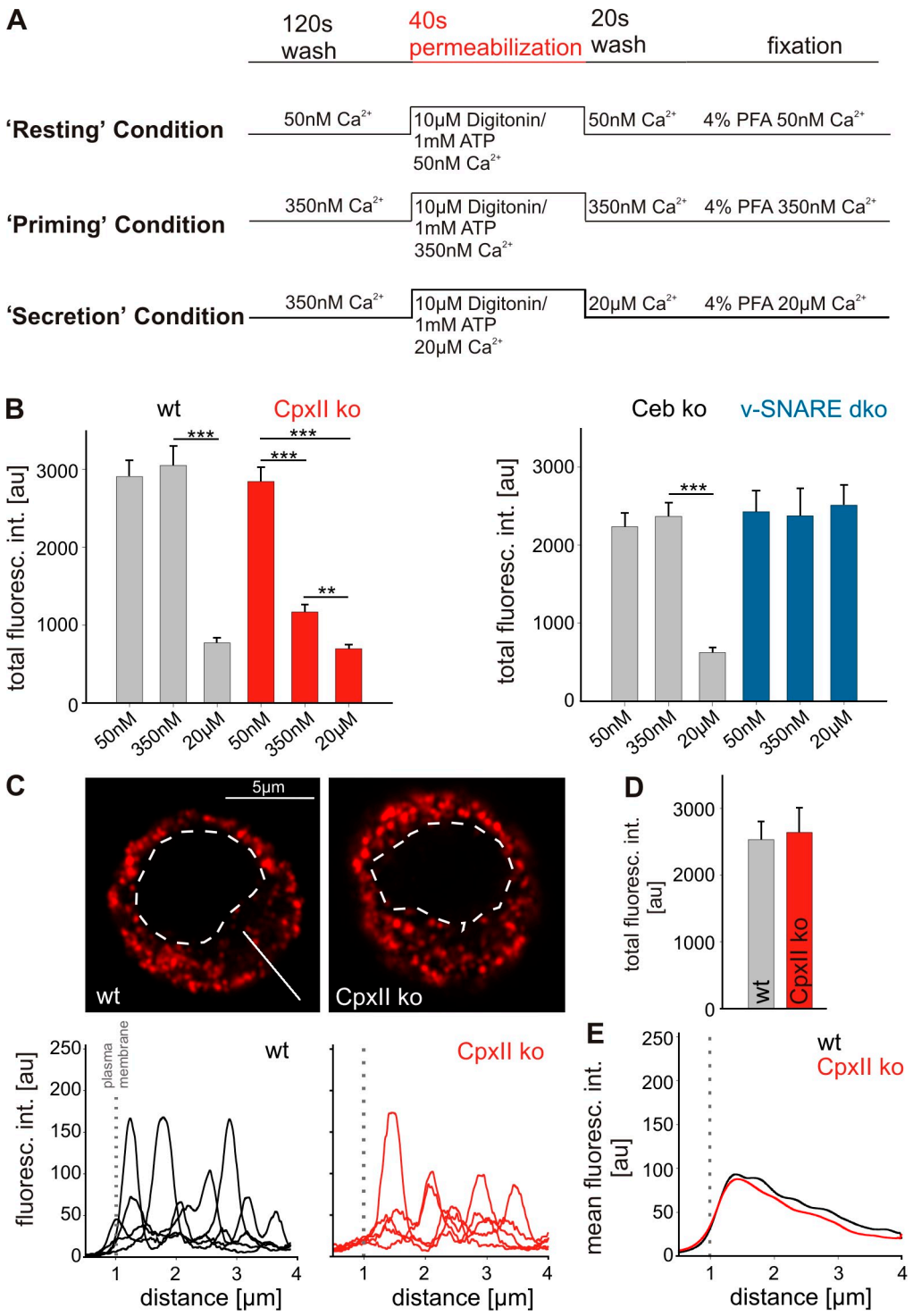

Figure S2. **Quantification of total fluorescence intensity for ChrgA in the cell cytoplasm confirms the significant loss of granules in CpxII ko cells at 350 nM [Ca]i.** (A) Experimental protocol for results shown in Fig. 2 and obtained at resting (50 nM), priming (350 nM), and secretion (20 µM) conditions of [Ca]i. Cells were permeabilized for 40 s with 10 µM digitonin in the presence of 1 mM ATP bracketed by washing with Ringer's solution at the indicated free [Ca]i. Cells were fixed with 4% PFA and processed for further immunostaining. (B) Quantification of the total fluorescence intensity of the cytoplasm (arbitrary units [au]) determined for the indicated genotypes at different [Ca]i. Neither loss of CpxII nor v-SNARE deficiency changes the ChgrA staining at 50 nM [Ca]i when compared with controls. At 350 nM [Ca]i, the ChgrA staining of CpxII ko cells is significantly decreased, indicating the loss of secretory vesicles, consistent with line scan analyses shown in Fig. 2. A strong reduction of the ChgrA signal is observed for wt, CpxII ko, and cellubrevin (Ceb) ko cells at 20 µM [Ca]i as a result of efficient granule exocytosis that is impaired in v-SNARE dko cells. Cellubrevin ko cells serve as littermate control for the v-SNARE dko cells. (C and D) Exemplary images of resting wt and CpxII ko cells (top) not treated with digitonin and their corresponding line scan analyses (bottom). The pattern and distribution of the ChgrA signal as well as the intensity of the total cytoplasmic fluorescence (D) resembles closely the staining of digitonin-treated cells exposed to 50 nM [Ca]i (Fig. 2), showing that digitonin treatment does not alter granule organization. (E) Mean line scans of wt (n = 20) and CpxII ko cells (n = 19) not treated with digitonin. ***, $P < 0.001$, one-way ANOVA followed by Tukey–Kramer post hoc test within the same genotype and Student's t test between genotypes. int., intensity. Error bars indicate means ± SEM.

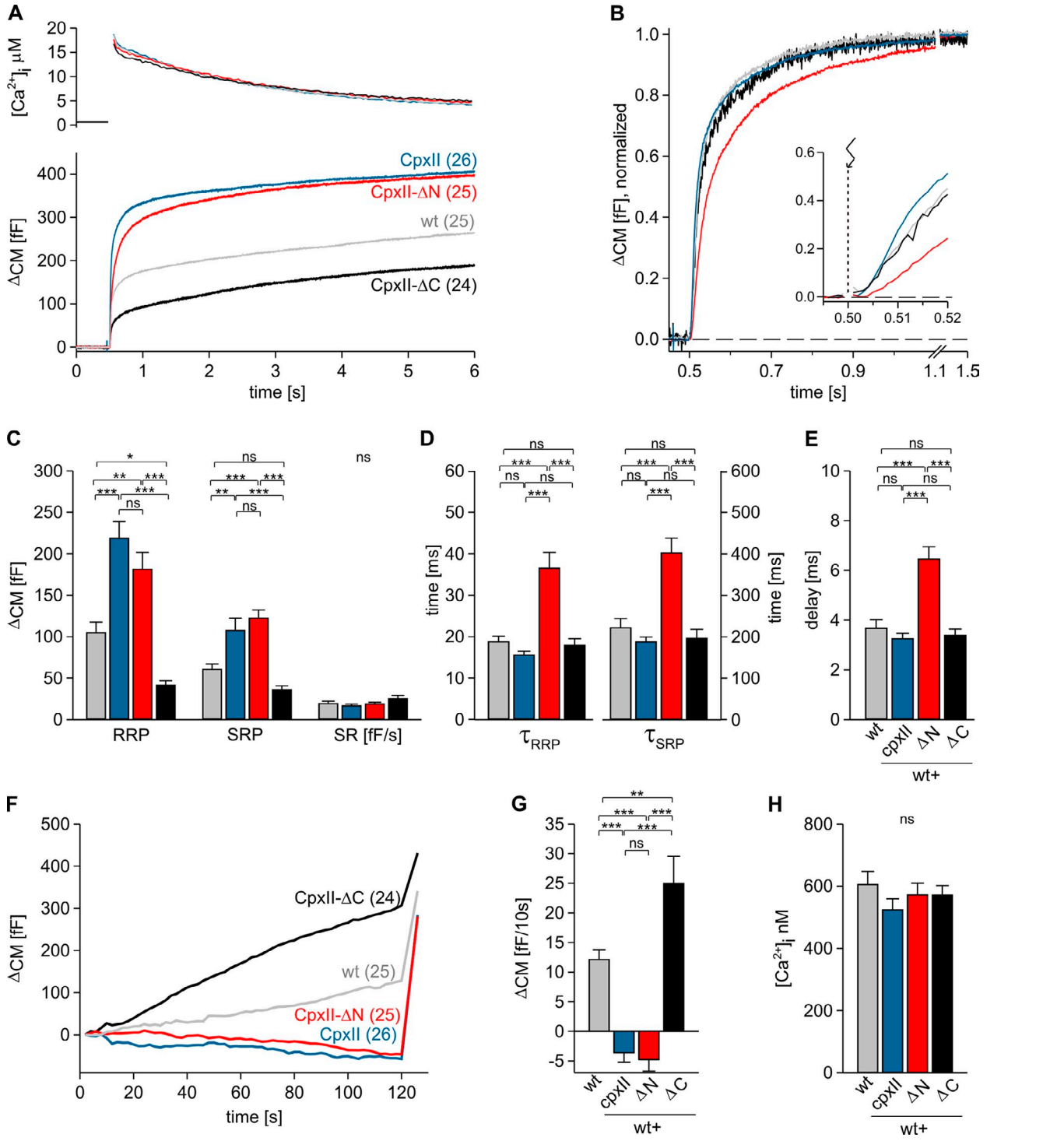

Figure S3. **The ΔC and ΔN mutants compete with endogenous CpxII, changing the magnitude and kinetics of synchronized exocytosis.** (A) Mean flash-induced $[Ca^{2+}]i$ levels (top) and corresponding capacitance responses (bottom) of wild-type (wt) cells (n = 25) and those expressing CpxII or its mutated variants. Both CpxII (wt + CpxII, n = 26) and the ΔN mutant (wt + CpxII-ΔN, n = 25) strongly increase the EB when compared with controls. Contrarily, the ΔC mutant (wt + CpxII-ΔC, n = 24) efficiently decreases the EB. Flash, t = 0.5 s. (B) Capacitance signals (as shown in A) normalized to the EB size 1 s after the flash (dashed line indicates the baseline). The ΔN mutant slows down the kinetics of the EB, whereas the ΔC mutant promotes synchronized exocytosis as fast as the wt response. The inset shows the extended scaling of this normalized capacitance signals during the first 20 ms after flash (arrow, dotted line). (C) Amplitudes of the two EB components (RRP and SRP) and the rate of sustained secretion (SR, femtofarad/second) for wt (gray bars), CpxII (blue bars), CpxII-ΔN (red bars), and CpxII-ΔC (black bars). CpxII-ΔN boosts RRP and SRP components of the EB, like CpxII, whereas the CpxII-ΔC mutant suppresses both phases of the burst. No significant changes of the sustained secretion rate are detected. (D) CpxII-ΔN slows down significantly the time constants of the burst (τ_{RRP} and τ_{SRP}). (E) The mean exocytotic delay determined from fitting individual cellular responses is significantly longer for secretion promoted by CpxII-ΔN. (F) Mean capacitance responses (same group of cells as shown in A) during infusion of submicromolar $[Ca^{2+}]i$. Before the uncaging flash, CpxII and its ΔN mutant clamp tonic exocytosis. In contrast, CpxII-ΔC efficiently unclamps secretion beyond levels recorded in wt cells. (G and H) Mean rate of tonic exocytosis determined at similar submicromolar $[Ca^{2+}]i$ (H) before the uncaging flash for the groups shown in G. *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA followed by Tukey–Kramer post hoc test. Error bars indicate means ± SEM.

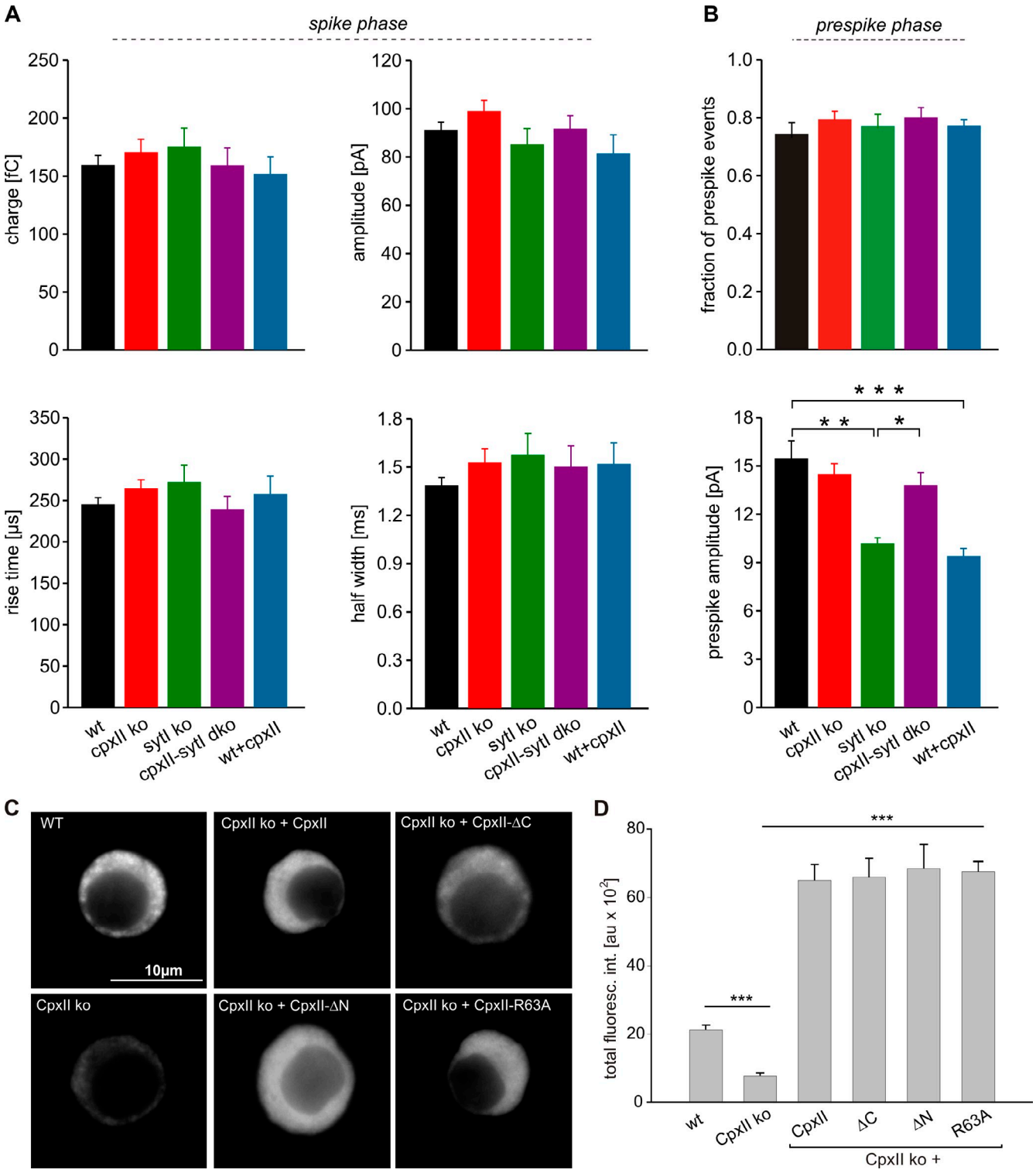

Figure S4. **Analysis of amperometric spike properties and of expression levels of CpxII wt and mutant proteins.** (A) Properties of amperometric events during spike phase averaged from wt (72), CpxII ko (38), SytI ko (22), CpxII-SytI dko (20), and wt + CpxII (31) cells. Data were collected from cells with >40 events. Notably, none of the spike phase parameters are altered in different mutants. (B) Fraction of events exhibiting prespike signals is unchanged in various genotypes. Along with increased prespike duration and charge (Fig. 7), the prespike amplitude is also decreased in SytI ko and wt cells overexpressing CpxII. Values are given in means (±SEM) for spike amplitude and initial foot amplitude. Other values are given as average medians determined from the parameter's frequency distribution for each cell. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ one-way ANOVA followed by Tukey–Kramer post hoc test between the indicated groups. (C) Exemplary images of wt, CpxII ko, and CpxII ko cells overexpressing either CpxII or the mutated CpxII proteins. Immunosignals were detected with a polyclonal CpxII antibody. Signals are visualized after adjustment of the camera's exposure time (wt, 310 ms; CpxII ko + CpxII and mutants, 91 ms). (D) Mean total fluorescence intensity of wt and CpxII ko cells expressing CpxII or the indicated mutants (determined 3.5 h after transfection). Note that CpxII mutant proteins do not differ from CpxII regarding the level of protein expression in CpxII ko cells (wt, $n = 15$; CpxII ko, $n = 16$; CpxII, $n = 14$; CpxII-ΔC, $n = 17$; CpxII-ΔN, $n = 16$; CpxII-R63A, $n = 17$). ***, $P < 0.001$, one-way ANOVA versus wt. Error bars indicate means ± SEM. au, arbitrary unit; int., intensity.

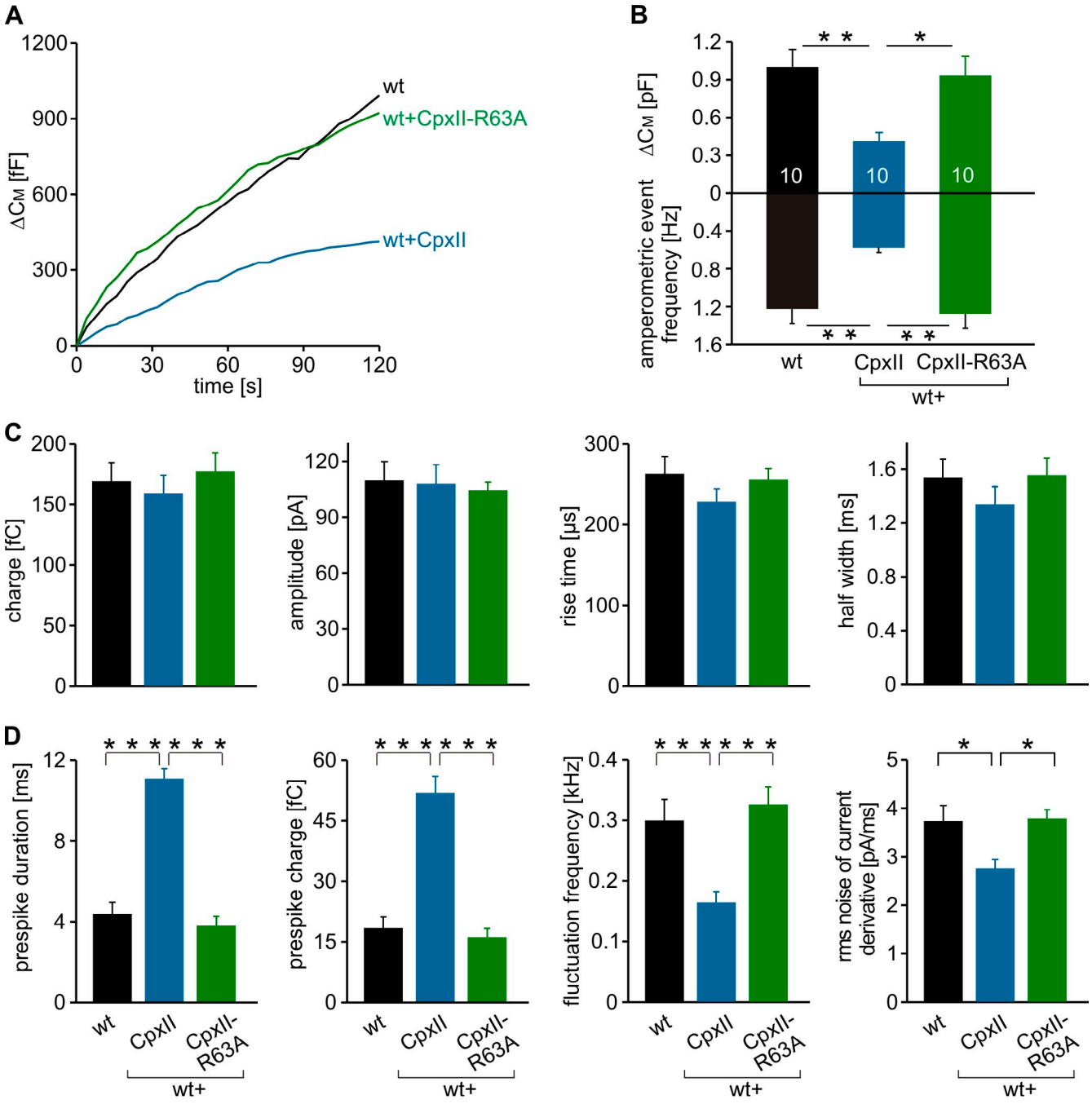

Figure S5. **The SNARE binding mutant CpxII-R63A fails to clamp secretion.** (A) Mean CM increase upon perfusion with 19 µM of free Ca^{2+} for 120 s in the indicated groups. (B) In contrast to the wt CpxII protein, the mutant variant CpxII-R63A fails to decrease tonic release, indicating that SNARE binding is important for CpxII to clamp tonic exocytosis. Data are averaged from the indicated number of cells. (C) Properties of amperometric events (>7 pA) during the spike phase. Data were collected from cells with >40 events. Notably, none of the spike phase parameters are altered after expression of CpxII and its mutant variant in wt cells. (D) Prespike signal duration and its charge are increased in CpxII but not in CpxR63A-expressing cells. Fluctuation frequency and rms noise of the current derivative during the prespike signal are reduced with CpxII but are unchanged with CpxII-R63A, indicating that SNARE binding of CpxII is similarly important for clamping the early fusion pore. Values are given as means (±SEM) for spike amplitude and initial foot amplitude. Other values are given as average medians determined from the parameter's frequency distribution for each cell. *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA followed by Tukey–Kramer post hoc test between the indicated groups.