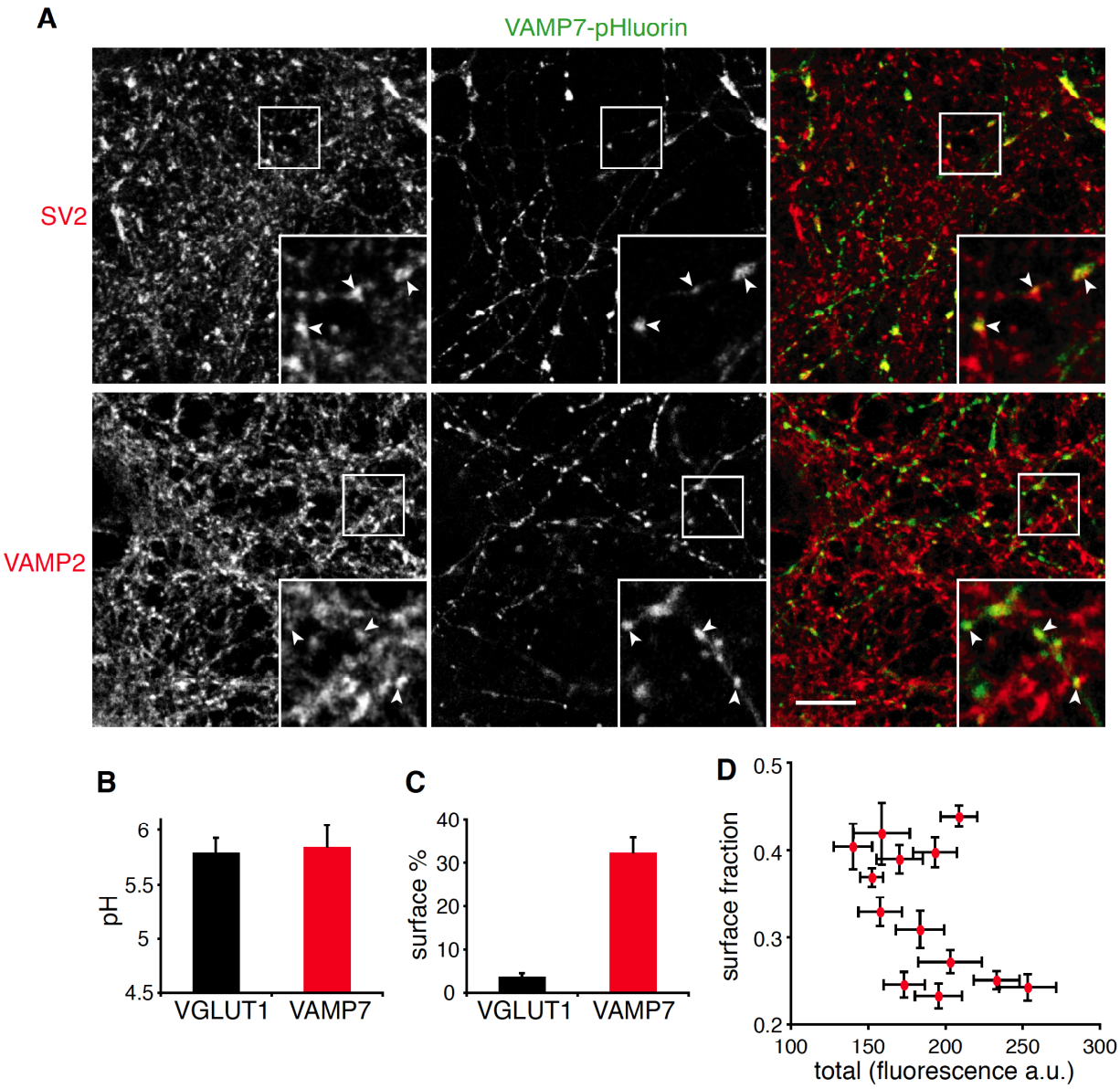

Figure S1. VAMP7-pHluorin localizes to presynaptic boutons by immunofluorescence, related to Figure 1

(**A**) Hippocampal neurons were transfected with VAMP7-pHluorin (green) and after fixation, double stained for endogenous SV2 or VAMP2 (red). Inset shows magnification of the area inside the box. Transfected VAMP7-pHluorin colocalizes in part with these presynaptic proteins (arrowheads). Size bar, 10 μm (**B**) Expressed in hippocampal neurons, VAMP7-pHluorin localizes to vesicles with a pH similar to VGLUT1-pHluorin. n = 3 coverslips containing a total of 135 boutons for VGLUT1 and 116 boutons for VAMP7 (**C**) VAMP7-pHluorin exhibits a higher surface expression level at steady state than VGLUT1-pHluorin ($p < 0.0001$). n = 7 coverslips containing 702-715 boutons per group (**D**) The surface fraction of VAMP7-pHluorin does not correlate with total expression level determined in NH_4Cl. The data indicate mean ± SEM for individual coverslips containing 50-100 boutons each.

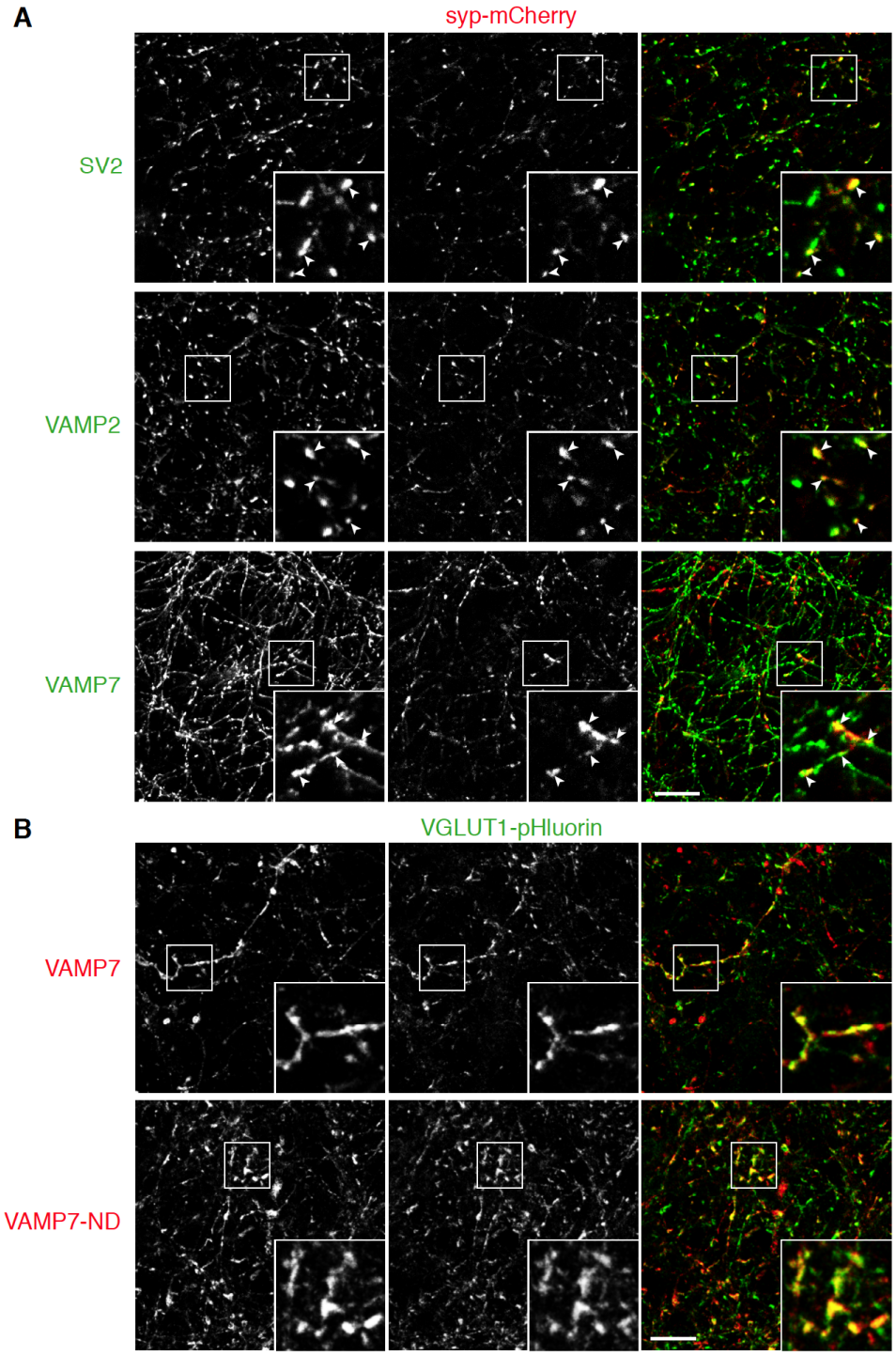

Figure S2. Localization of transfected synaptic vesicle proteins to presynaptic sites, related to Figure 2

(**A**) Hippocampal neurons were transfected with synaptophysin-mCherry (syp-mCherry) and double stained for endogenous SV2, VAMP2 and VAMP7 (green). Inset shows a magnification of the area inside the box, and arrowheads indicate boutons where syp-mCherry colocalizes with the endogenous synaptic vesicle proteins.

(**B**) Neurons were cotransfected with VGLUT1-pHluorin (green) and untagged VAMP7, either wild type (top) or the ND mutant (bottom), and immunostained for VAMP7 (red) using an antibody that cannot detect the low endogenous levels of VAMP7 (data not shown). Both wild type and mutant VAMP7 colocalize extensively with VGLUT1-pHluorin. Size bar, 10 μm

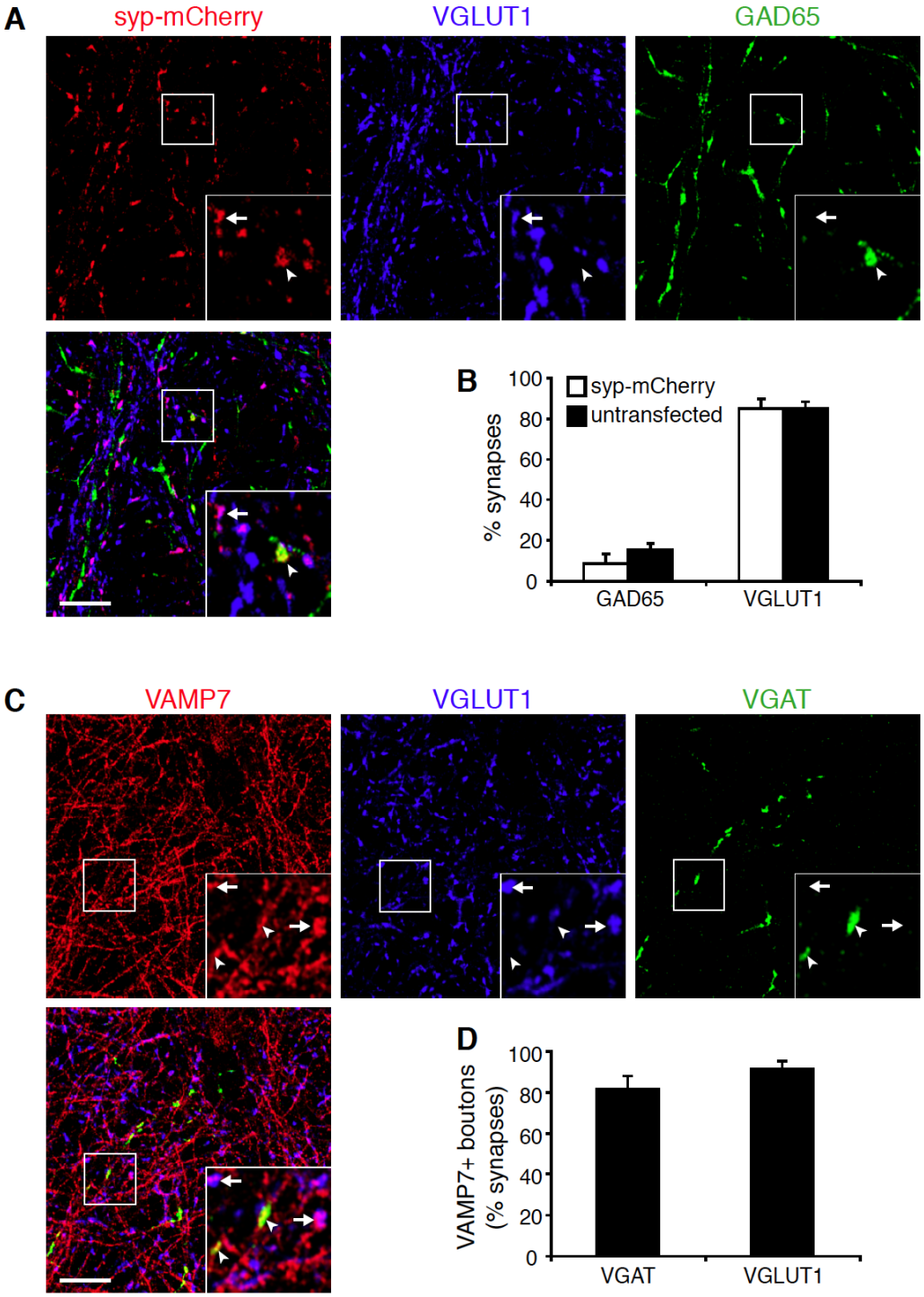

Figure S3. Syp-mCherry and VAMP7 localize to both excitatory and inhibitory synapses, related to Figure 3

(**A**) Hippocampal neurons were transfected with syp-mCherry and double stained for endogenous VGLUT1 (blue) and GAD65 (green). The arrow indicates colocalization of syp-mCherry with VGLUT1, and arrowhead with GAD65. (**B**) Syp-mCherry targets to excitatory (VGLUT1+) and inhibitory (GAD65+) synapses in the same proportion observed for these boutons in the untransfected population. (**C**) Neurons were stained for endogenous VAMP7 (red), VGLUT1 (blue) and VGAT (green). Arrows indicate colocalization of VAMP7 with VGLUT1, and arrowheads with VGAT. (**D**) VAMP7 localizes to the majority of both excitatory (VGLUT1+) and inhibitory (VGAT+) synapses. Size bar, 10 μm

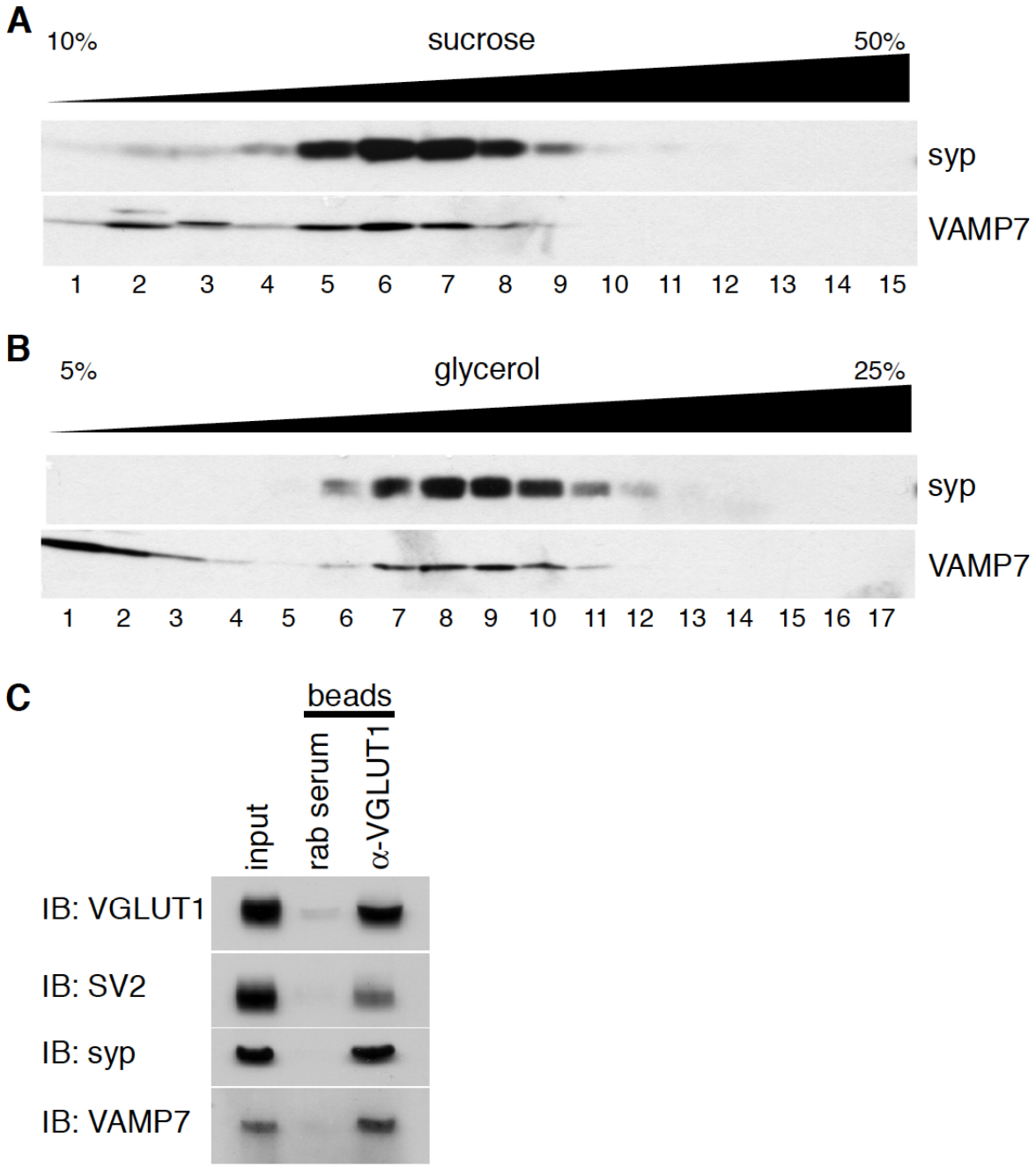

Figure S4. VAMP7 localizes to synaptic vesicles, related to Figure 4
(**A**,**B**) A cell lysate from cultured hippocampal neurons was sedimented at 1000,000g•min, the supernatant subjected to equilibrium sedimentation through sucrose (**A**) or velocity sedimentation through glycerol (**B**), and the fractions immunoblotted for synaptophysin (syp) and VAMP7. VAMP7 distributes in two peaks, one of which coincides with syp. (**C**) Immunoisolation with an antibody to VGLUT1 yields synaptic vesicles that contain VAMP7 as well as SV2 and synaptophysin (syp).

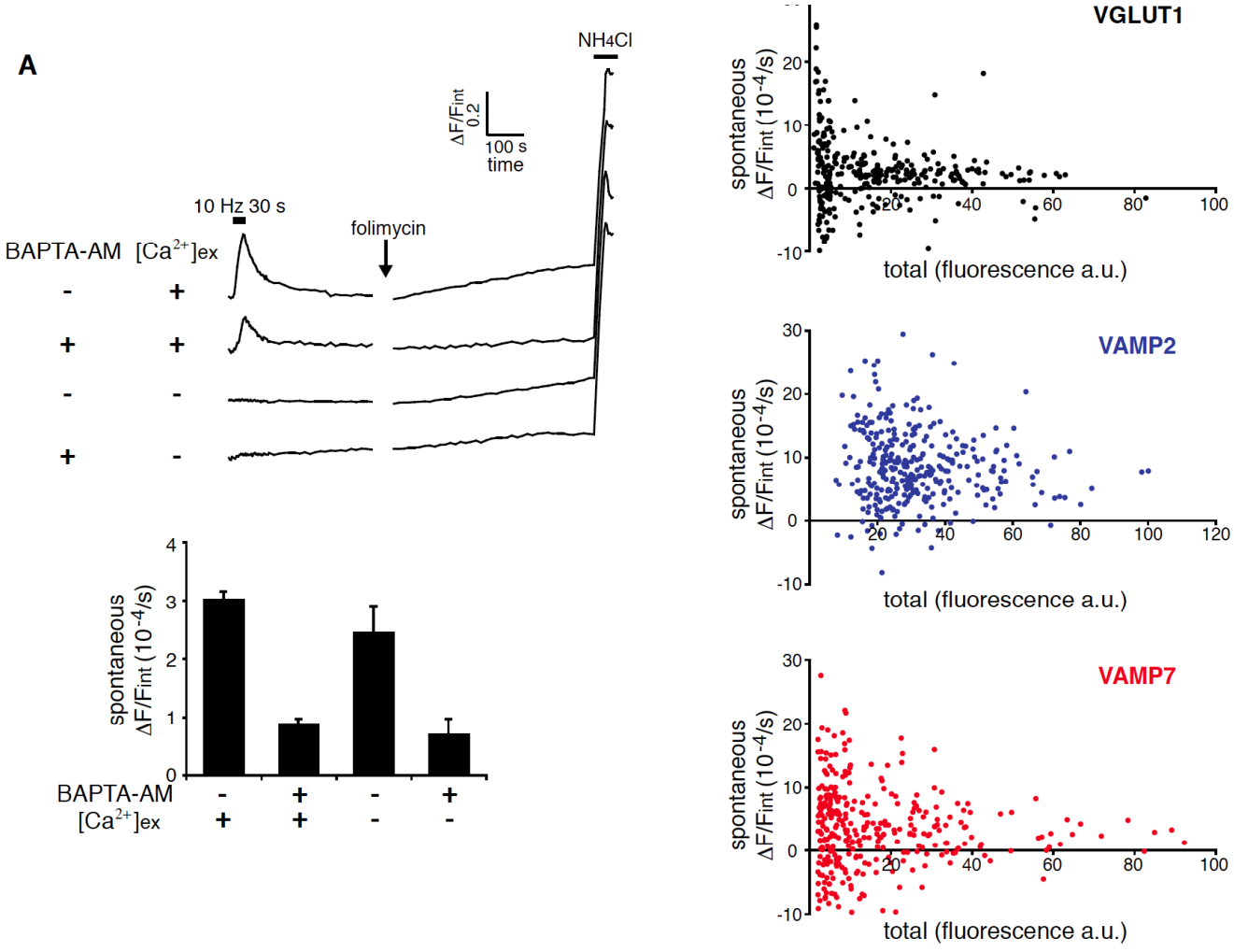

Figure S5. Measurement of spontaneous release using folimycin, related to Figure 5
(**A**) Hippocampal neurons expressing VGLUT1-pHluorin were stimulated at 10 Hz for 30 s, treated with folimycin 5 minutes later, and total fluorescence revealed with NH_4Cl. The cells were treated either with or without BAPTA-AM, and imaged in Tyrode's buffer with either 2 mM Ca^{++} or 0 mM Ca^{++} (with 0.5 mM EGTA). Removing external Ca^{++} abolished the evoked response, but only slightly reduced the spontaneous increase in fluorescence. Chelation of intracellular Ca^{++} with BAPTA-AM reduced the spontaneous increase in fluorescence. Quantitation of the spontaneous fluorescence increase demonstrates its particular sensitivity to BAPTA-AM, thus excluding cell permeation by folimycin and the alkalinization of intracellular vesicles that have not undergone exocytosis. n = 3 coverslips containing a total of 171-202 boutons per group (**B**) Spontaneous releases does not correlate with the expression of pHluorin (assessed in NH_4Cl) for VGLUT1, VAMP2 or VAMP7 fusions. n = 5-6 coverslips containing 328-366 boutons for each construct

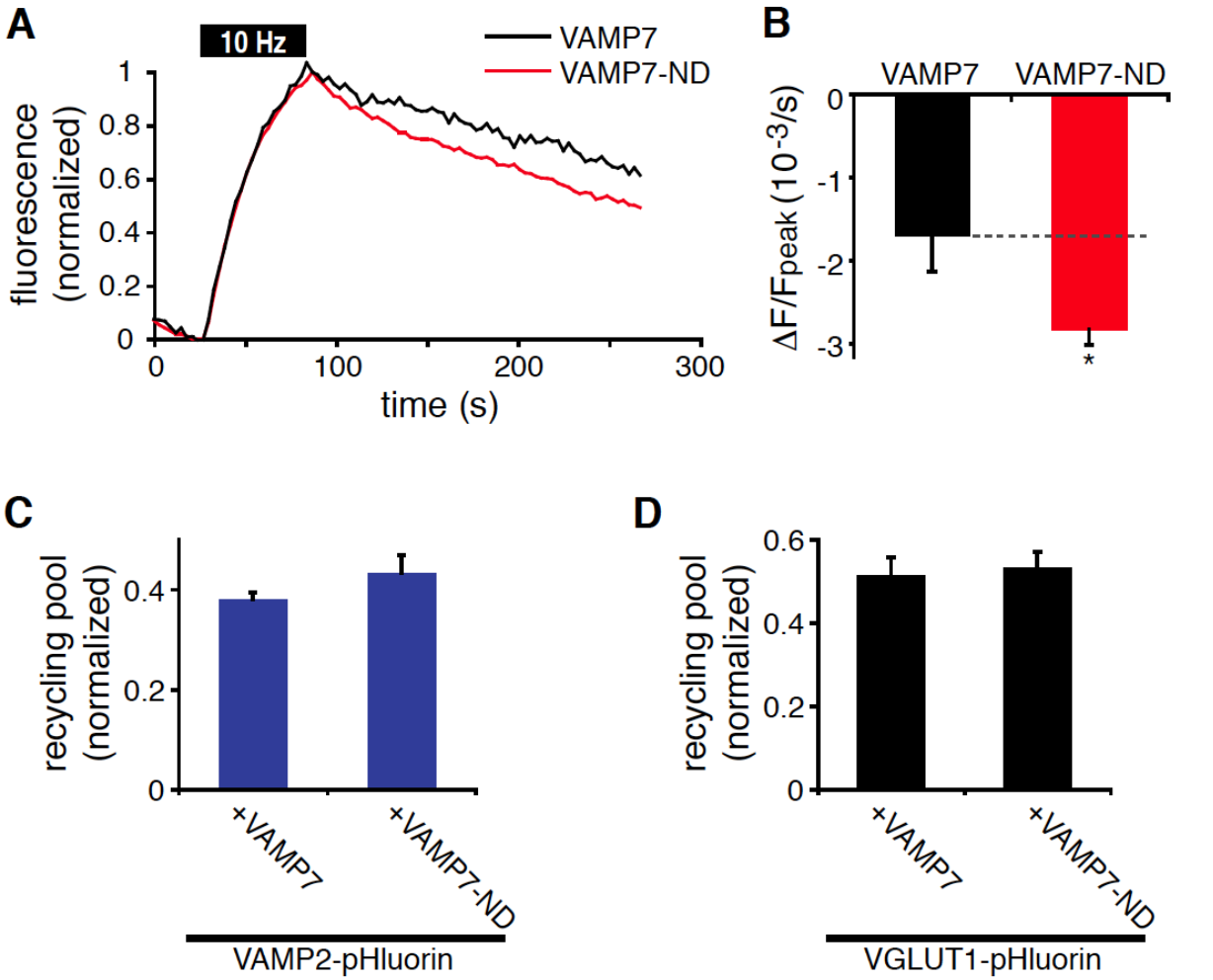

Figure S6. VAMP7 lacking the longin domain does not affect the evoked release of VAMP2 or VGLUT1, related to Figure 7

(**A**) Hippocampal neurons transfected with VAMP7- or VAMP7-ND-pHluorin were stimulated at 10 Hz for 60 s. (**B**) The rate of VAMP7-ND endocytosis (determined by linear fit) is faster than that of wild type VAMP7. n = 6 coverslips containing 335-345 boutons per group. (**C**) Coexpression of untagged VAMP7-ND does not affect the proportion of VAMP2-pHluorin in the recycling pool. n = 3 coverslips containing 170-173 boutons per group (**D**) Cotransfection of VAMP7-ND does not affect the proportion of VGLUT1-pHluorin in the recycling pool. n = 6 coverslips containing 337-353 boutons per goup