Supplementary Methods

Photobleaching for CLEM experiments

In order to effectively remove all FM1-43 derived photoconversion products in the bleach region of CLEM experiments, we scanned our rectangular region(s) of interest ten times at maximal laser power. In samples fixed immediately after photobleaching > 99% of vesicles in the bleach region of serial sections were PC− (Fig. 3a). We established that the bleach protocol was not detrimental to bouton health by demonstrating their ability to take up and then release FM1-43 following photobleaching (Supplementary Fig. 2).

Electron micrograph analysis

Vesicle numbers were quantified by first defining the lateral limits of the presynaptic terminal for each synapse, using the section with the longest continuum of vesicles lying successively within 100 nm of one another, in a plane longitudinal to the axon. This boundary was then applied to all other sections of the synapse. This proved to be a satisfactory definition of the vesicle cluster which took into account its three-dimensional organization. Vesicles (n = 7878 total), excluding vesicle-like structures exceeding 55 nm in diameter, were scored as PC+ or PC− based on a visual assessment of their lumenal intensities. In vesicles where this classification was unclear, we measured the ratio of lumenal to membrane density as described previously (Rizzoli SO, Betz WJ, Science 2004, 303:2037-9). Analyzing a subset of our data (n = 913 vesicles from 3
boutons) by this method revealed a bimodal distribution in density histograms with peaks at 0.9 and 1.2, corresponding to empty and full vesicles respectively (Fig. 2). Therefore, we defined full vesicles as those with a relative density of > 1.075. As a further confirmation of our scoring of PC+ and PC− vesicles a random selection of the data (n = 2682), including samples from all experimental conditions, was analyzed blind by a third party and this did not yield significantly different results. Samples that were photobleached and fixed immediately, by non-blind and blind counts contained 0.6 ± 0.4% and 0.4 ± 0.4% PC+ vesicles, respectively (t-test, P = 0.502). In samples that were jasplakinolide-treated, photobleached and fixed after 18 min, non-blind and blind counts yielded 2.3 ± 0.7% and 2.5 ± 1.1% PC+ vesicles, respectively (t-test, P = 0.840). Samples that were photobleached and fixed after 18 min, by non-blind and blind counts contained, 7.5 ± 1.6% and 9.2 ± 2.1% PC+ vesicles, respectively (t-test, P = 0.105). In non-photobleached samples, non-blind and blind counts yielded 44.6 ± 7.2% and 53.2 ± 9.7% PC+ vesicles, respectively (t-test, P = 0.079). The spatial organization of vesicles in each synapse was studied using two methods, regional analysis and measurement of distance to active zone, using three sections centered around the active zone for each synapse. For regional analysis we defined three regions of the vesicle cluster, active zone, core and edge, for each of the three sections. The active zone region was defined as a compartment extending 100 nm from the active zone into the vesicle cluster. The edge region was a single continuous line of the outermost vesicles each lying within two vesicle diameters (100 nm) of one another, starting with the vesicle
lying furthest, but < 100 nm, from the active zone membrane. Vesicles lying within 150 nm of this defined cluster edge were included in the edge region groups. The core region was that between the active zone and edge regions. For distance to active zone analysis we defined the position of every vesicle in three-dimensional space and measured the distance of each one to its nearest point along the active zone, making the assumption that each vesicle lay in the center of the section’s depth (60 nm). For serial reconstructions, a digital overlay, marking the positions of vesicles and active zones, was generated for each of the aligned sections. We assumed a vesicle diameter of 50 nm, based on the average diameter of our vesicle population. The overlays were then stacked in sequence and reconstructed using three-dimensional rendering software (Volocity, Improvision).