Supplementary Figure Legends

Supplementary Figure 1. The relative increase in ΔFRET or decrease in current after two stimuli is smaller for postsynapses and NMJs with higher transmission.

(**a**) The bigger the ΔFRET response for a single stimulus was, the smaller the relative increase in ΔFRET after two stimuli that summated ($r = 0.37$, $p = 0.017$, $n = 41$ boutons and 5 NMJs). This dependence is probably due to presynaptic depression as evoked synaptic currents (**b**) showed a similar relationship ($r = 0.34$, $p = 0.037$, $n = 38$ NMJs).

Supplementary Figure 2. GluR identity and distribution cannot explain the proximal-distal gradient of transmission strength.

(**a**) Immediately after imaging, larvae were fixed and stained for DGluRIIA and DGluRIIB. (**b**) The total amount of DGluRIIA, (**c**) DGluRIIB, or (**d**) the ratio DGluRIIA to DGluRIIB per bouton, as measured by the total intensity of antibody stain against each subunit, show little or no correlation to the average SynapCam FRET response of a postsynapse (correlation coefficients and p values for each, respectively $r = -0.082$, $p = 0.368$; $r = 0.166$, $p = 0.066$; $r = 0.183$, $p = 0.043$). Similarly (**e**) the density of DGluRIIA patches per bouton has only a weak correlation ($r = 0.213$, $p = 0.018$). Of all of these measures, at most, for a 10-fold difference in SynapCam FRET response there is a 0.5-fold difference in GluRs. This suggests that although GluR quantity and identity contribute to the size of the postsynaptic response they do not determine gradient of transmission strength. (**b-d**) Data consist of 124 postsynapses from 10 NMJs, each NMJ represented by a different symbol. For comparison, values were normalized within each NMJ dataset.

Supplementary Figure 3. Differences between boutons in the number of active zone patches or the total quantity of an active zone marker cannot explain the proximal-distal gradient of transmission strength.

(**a**) After imaging, active zones were identified with the Nc82 antibody[42] (in green, SynapCam3.1 YFP in red). (**b, c**) The total quantity of active zone marker, Nc82 and the density of Nc82 puncta per bouton showed no significant correlation to the mean ΔFRET amplitude (respectively, $r = 0.121$, $p = 0.384$; $r = 0.225$, $p = 0.102$). For all panels, data consist of 55 postsynapses from 5 NMJs, each NMJ represented by a different symbol. For comparison, values were normalized within each NMJ dataset.

Supplementary Figure 4.

(**a**) The results indicate the existence of a presynaptic gradient that runs along axonal branches, where the most distal boutons are the most potent, and there is a progressive decrease in transmission strength toward the branch origin. (**b**) This gradient may be established by retention of an inhibitor of transmission at branch origins or by active transport of an enhancer of transmission toward branch termini.