A resting pool of vesicles is responsible for spontaneous vesicle fusion at the synapse

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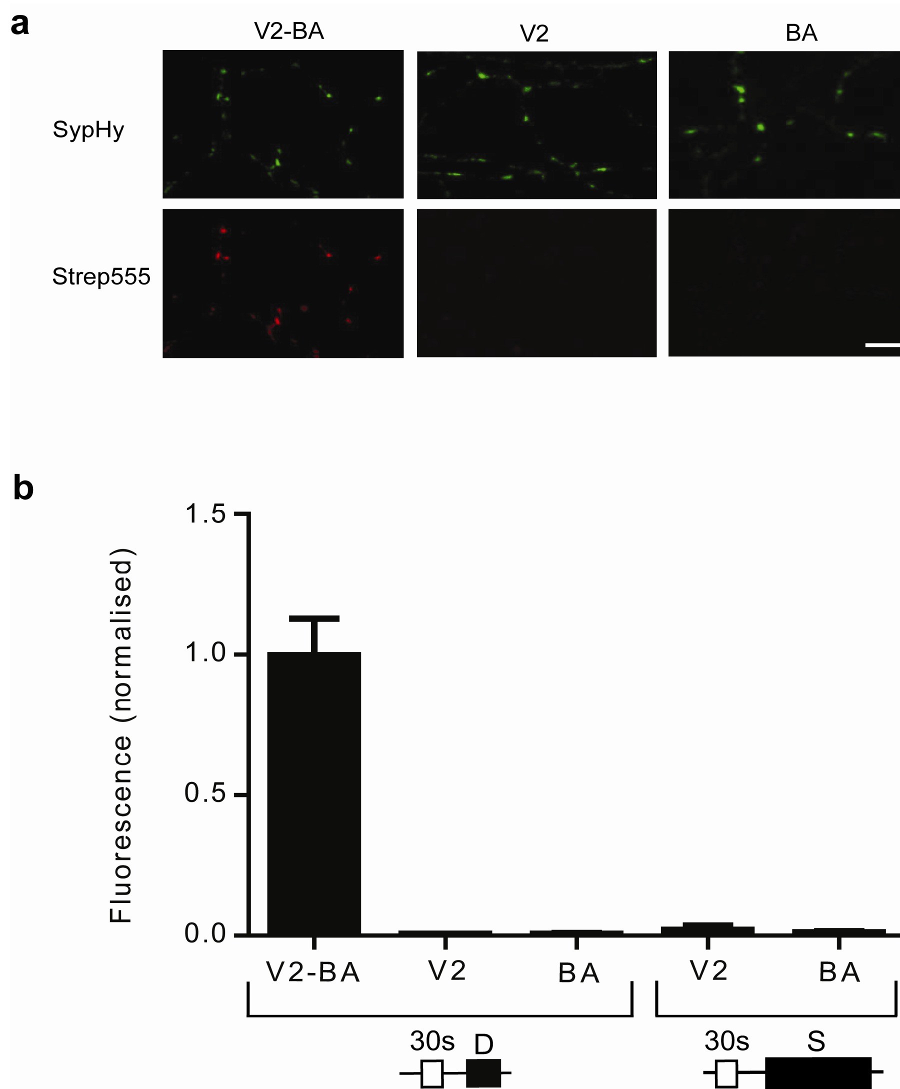
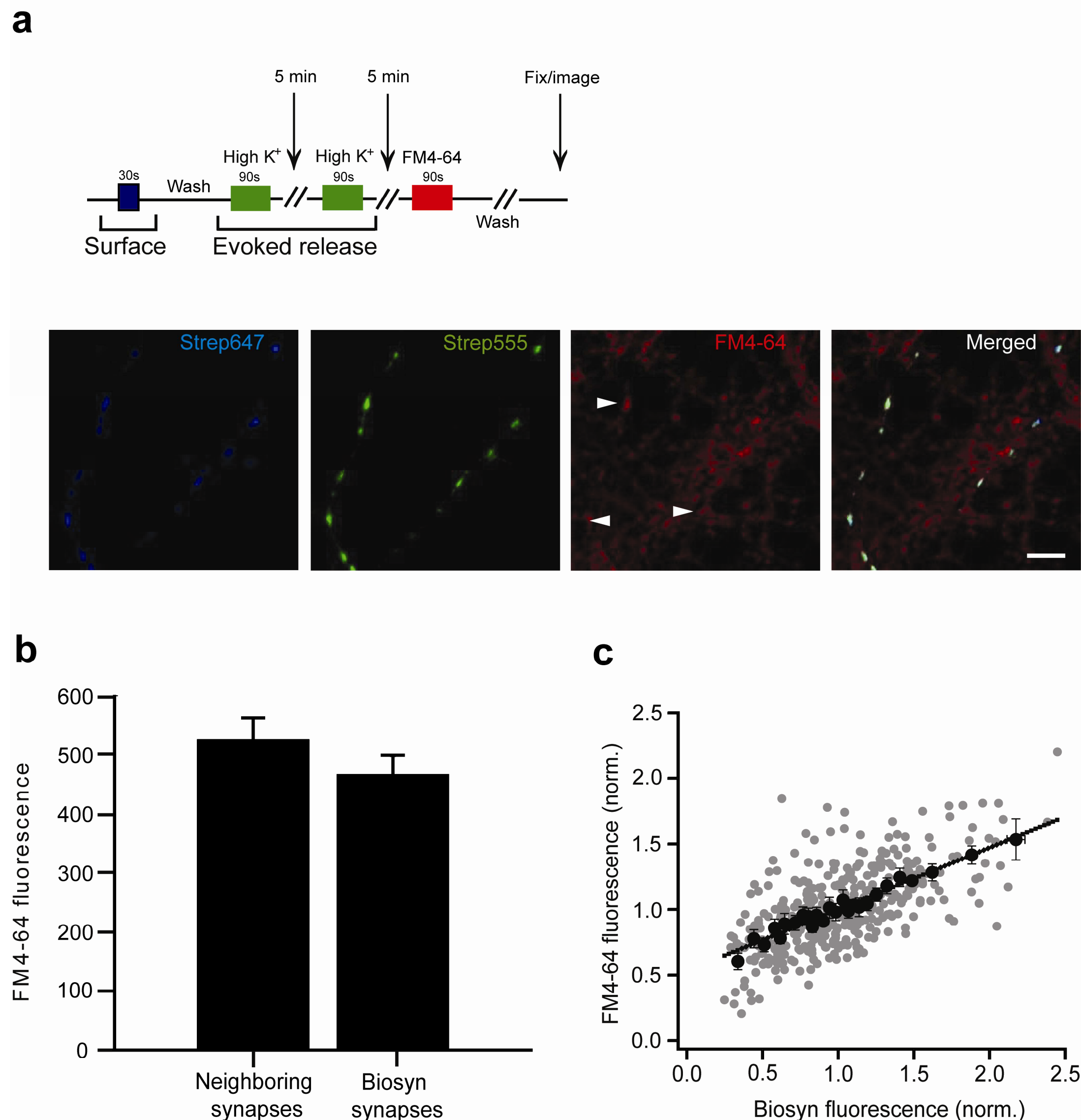
Supplementary Figure 1



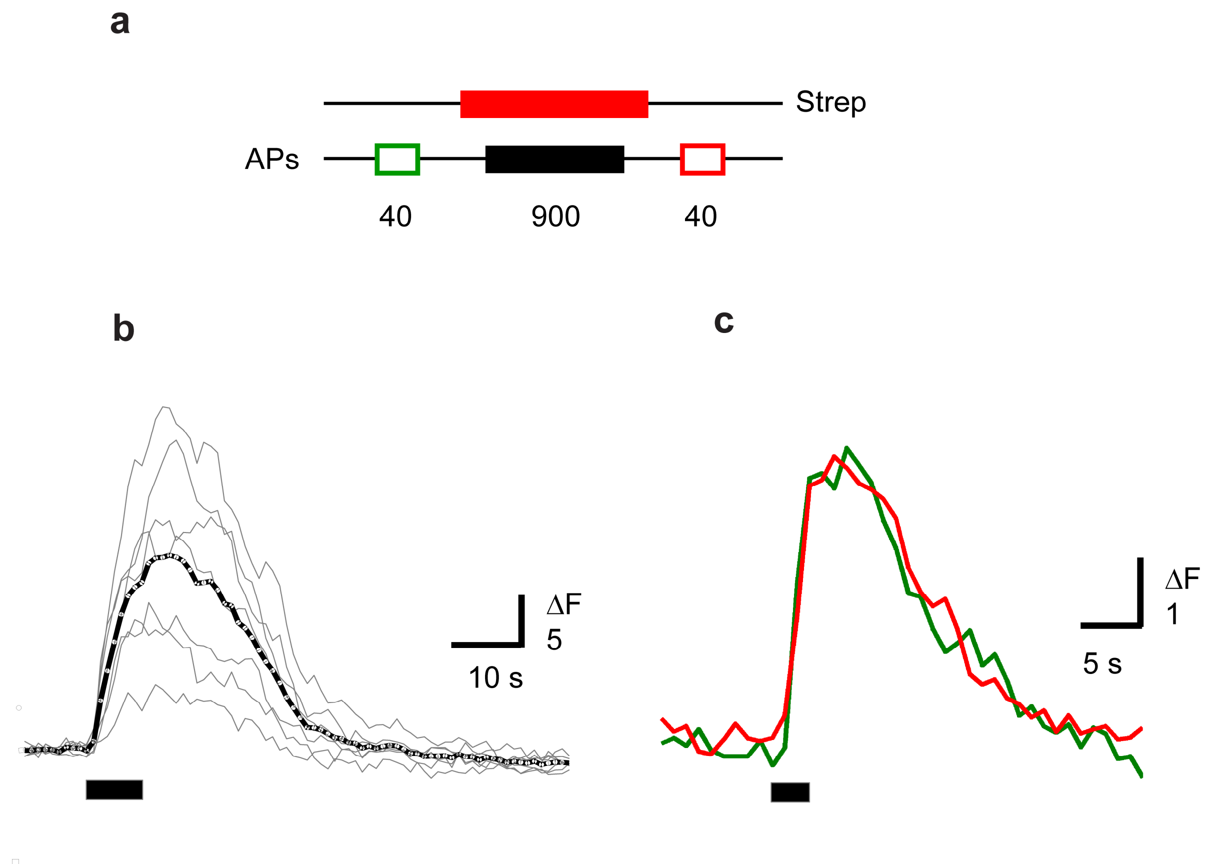
Specificity of the biosyn probe. (**a**) Neurons expressing sypHy, Vamp2-BAP (V2) and BirA (BA) showed strong labelling with Strep555 after two consecutive high K^+ stimuli (left). Neurons transfected with sypHy and either VAMP2-BAP (middle) or BirA (right) alone did not show any staining. Scale bar: 5 µm. (**b**) The graph shows the pooled fluorescence intensity data of synapses labelled with a depolarizing stimulus (D) or during spontaneous release (S) in the presence of strep555. Fluorescence values were normalized to the size of the recycling pool. The schematics below show the protocol followed in each case. Open bars represent 30 seconds of surface staining with unlabelled streptavidin; closed bars represent staining with strep555. Neurons were transfected in three different ways: with VAMP2-BAP and BirA (V2-BA), with VAMP2-BAP only (V2) and with BirA only (BA). Note that strong labelling is only observed when both VAMP-2 and BirA are co-expressed (V2-BA). When either one is expressed independently (V2 or BA) no labelling was observed after high K^+ stimulation or during 15 min of spontaneous cycling at 37° C.

Supplementary Figure 2



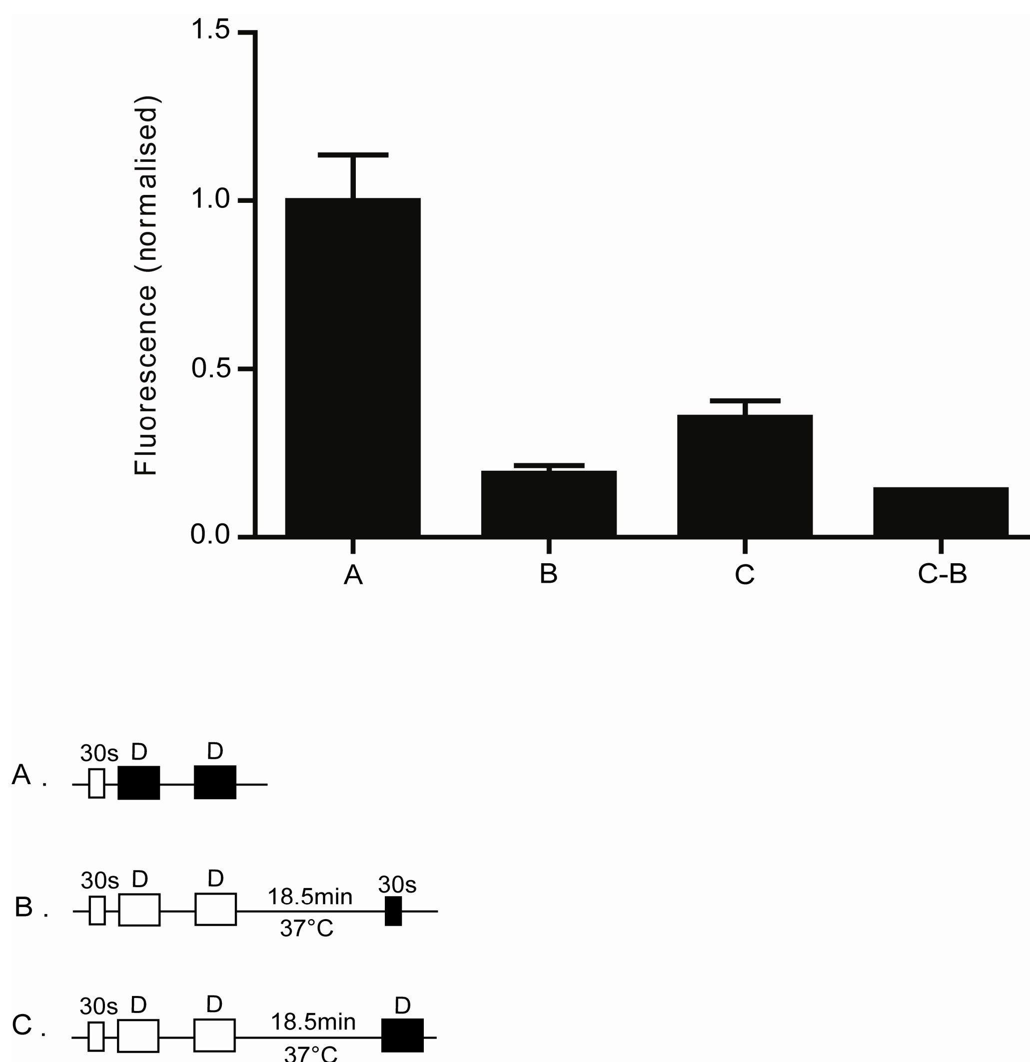
FM dyes show that biosyn labelling does not affect vesicle cycling. (**a**) Example images of presynaptic terminals where the surface labelling of biosyn is shown in blue and the labelling in response to two high K^+ depolarizations with strep488 is shown in green. The same synapses where subsequently depolarized with a single high K^+ depolarization in the presence of FM4-64 as shown in red. White arrows indicate example synapses clearly labelled with strep488 and also stained with FM4-64. (**b**) Average fluorescence intensity of FM4-64 puncta for synapses labelled with biosyn and neighbouring, unlabelled synapses, showing no significant difference between the two groups (unpaired T-test p = 0.2377). (**c**) Graph showing fluorescence intensity of FM4-64 staining as a function of biosyn labelling (gray dots are individual synapses; black circles are binned averages). A strong correlation exists between biosyn and FM4-64 staining, which provides further evidence that the same set of vesicles are being reused normally, after labelling with streptavidin-alexa. The dotted line represents the best linear fit to the data.

Supplementary Figure 3



SypHy shows that biosyn labelling does not affect vesicle cycling. (**a**) Schematic diagram showing the time line of the experimental protocol: neurons were first stimulated with 40APs at 20Hz (open green box), followed by 900APs at 20 Hz (black box) in the presence of strep555 to label biosyn. After washing to remove excess streptavidin, neurons were once again stimulated with 40 APs at 20 Hz (open red box). (**b**) Responses to 900 APs at 20 Hz (black bar) measured from all synapses analysed from a single cell (gray) and the average response overlayed in black. (**c**) Overlay of the responses to 40 APs at 20 Hz (black bar) before (green) and after (red) labelling the entire recycling pool with streptavidin. Note the amplitude and rates of exocytosis and endocytosis are very similar.

Supplementary Figure 4



The recycling pool and the spontaneous pool do not mix within the time-course of the experiment. The graph shows the fluorescence intensity (normalized to the recycling pool) that results from the staining protocols shown in the diagrams below (A to C). A. represents the fluorescence of the recycling pool. B. is the fluorescence that is left on the surface of the plasma membrane after 18.5 minutes (15 min at 37 C plus washes) of spontaneous recycling, having previously labelled the entire recycling pool as in A. At the end of the 18.5 min period the surface biosyn is labelled (30 s). The amount of staining observed represents the biosyn left behind on the surface of the plasma membrane during spontaneous vesicle cycling. C. represents the amount of evoked release that can be elicited at the same time-point as B. The amount of staining observed represents the sum of the surface biosyn plus the amount released through neuronal activity. Subtracting B from C gives the amount of release elicited by neuronal activity only, which is shown as the final bar in the graph (C-B). The total amount of activity-dependent vesicles available does not recover during the spontaneous recycling period at 37° C. Our data clearly show that two high K^+ stimulations release most of the recycling pool and that this pool depletion is constant throughout the entire period of spontaneous labelling.