A heterogeneous "resting" pool of synaptic vesicles that is dynamically interchanged across boutons in mammalian CNS synapses

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Using pHluorin-tagged synaptic vesicle proteins we have examined the partitioning of these probes into recycling and nonrecycling pools at hippocampal nerve terminals in cell culture. Our studies show that for three of the major synaptic vesicle components, vGlut-1, VAMP-2, and Synaptotagmin I, ~50–60% of the tagged protein appears in a recycling pool that responds readily to sustained action potential stimulation by mobilizing and fusing with the plasma membrane, while the remainder is targeted to a nonrecycling, acidic compartment. The fraction of recycling and nonrecycling (or resting) pools varied significantly across boutons within an individual axon, from 100% resting (silent) to 100% recycling. Single-bouton bleaching studies show that recycling and resting pools are dynamic and exchange between synaptic boutons. The quantitative parameters that can be extracted with the approaches outlined here should help elucidate the potential functional role of the resting vesicle pool.

Introduction

At nerve terminals, synaptic vesicles recycle to sustain neurotransmitter release during repetitive action potential (AP) firing. The size of the pool of vesicles that recycle and the kinetics of each of the recycling steps all play important roles in determining the efficacy of synaptic function during repetitive stimulation (Betz and Angleson, 1998; von Gersdorff and Matthews, 1999; Fernandez-Alfonso and Ryan, 2006). Traditionally, vesicle pools at synaptic terminals have been defined based on kinetic criteria related to their depletion rates (Elmqvist and Quastel, 1965; von Gersdorff and Matthews, 1997; Dobrunz and Stevens, 1997; Wu and Betz, 1998). The readily releasable pool is thought to correspond to vesicles in immediate apposition to the active zone (so-called docked vesicles) that undergo exocytosis during a small number of AP stimuli. Beyond the active zone, presynaptic terminals have clusters of dozens to hundreds of synaptic vesicles that are recruited for exocytosis during prolonged stimulation and are referred to as the reserve pool (Stevens and Tsujimoto, 1995; Pieribone et al., 1995; Neher, 1998). The entire recycling vesicle pool is comprised of both the readily releasable and reserve pool of vesicles. FM dye labeling of synaptic vesicles in hippocampal nerve terminals show that the entire pool of recycling vesicles can be turned over with a few hundred stimuli, i.e., the amount of FM loaded saturates after a few hundred stimuli, and a similar number of stimuli causes >90% of the dye to be released (Ryan and Smith, 1995;

Ryan et al., 1996). However, the number of vesicles loaded with such protocols appears significantly lower than those independently observed in electron micrographs (Murthy et al., 1997; Murthy and Stevens, 1999). Additionally, ultrastructural localization of FM dyes, following photoconversion to an electron dense product, shows that only a fraction of the vesicles appear to have acquired dye even after loading under maximal stimulation (Harata et al., 2001). Recently, using synapto-pHluorin to label synaptic vesicles, Li et al. (2005) suggested that some vesicles existed in a resting pool that would not fuse with the plasma membrane upon strong stimulation; however, a detailed quantification of this pool or its dynamics across boutons and during long time scales was not provided. The simplest interpretation of these data is that a portion of the vesicle pool fails to recycle (Murthy et al., 1997; Murthy and Stevens, 1999; Sudhof, 2000). Similar observations have been made in the Calyx of Held, a giant auditory brain stem synapse, where exhaustive FM labeling resulted in only a minor fraction of ultrastructurally identifiable synaptic vesicle labeling (de Lange et al., 2003). Here, we have sought to examine the characteristics of both recycling and resting vesicle pools in a quantitative fashion in live cells. We made use of pHluorin-tagged synaptic vesicle proteins and the pH sensitivity of these probes to determine when and if they can be mobilized to fuse with the plasma membrane at synaptic terminals.

pHluorin is a modified form of GFP whose pH sensitivity has been shifted relative to that of EGFP by one log unit such that the pK_a is ~7.1 (Sankaranarayanan et al., 2000). Upon binding the protons the fluorescence is completely quenched, and therefore the fluorescence reflects the fraction of the time the fluorescent protein is in the proton-bound state. pHluorins can be targeted to the lumen of synaptic vesicles by fusion to one of the ~10 identified synaptic vesicle proteins with membrane spanning domains (Takamori et al., 2006). When expressed in neurons these proteins provide an useful readout of synaptic exocytosis and synaptic vesicle recycling. To date, four of the most abundant synaptic vesicle proteins, VAMP-2 (Miesenbock et al., 1998), vGlut-1 (Voglmaier et al., 2006), Synaptotagmin I (Fernandez-Alfonso et al., 2006), and Synaptophysin (Granseth et al., 2006) have been successfully used for examining

cycles of exo-endocytosis at nerve terminals with pHluorin tagging.

During synaptic vesicle recycling, the lumen of the vesicle is reacidified by virtue of the action of a V-type ATPase that develops a pH gradient and the proton motive force providing the energy to drive neurotransmitter uptake. When an inhibitor of this proton pump is applied to synapses during repetitive stimulation, vesicles continue to recycle; however, they fail to reacidify and remain alkaline after endocytosis (termed alkaline trapping, Sankaranarayanan and Ryan, 2001). Resting vesicles remain acidic and can be quantified by use of NH_4Cl that can rapidly alkanalize internal organelles. Comparison of the steady-state level of fluorescence achieved with pHluorin-tagged vesicle proteins during prolonged AP stimulation and alkaline trapping, with that achieved by direct alkalization of the entire vesicle pool provides a real time estimate of the fraction of the total pool of these vesicle proteins that remain acidic and hence fails to recycle.

Using these approaches, we show that a variable fraction of the total pool of the synaptic vesicle proteins VAMP-2, vGlut-1, and Synaptotagmin I do not participate in recycling during repetitive stimulation, but are dynamically exchanged between synapses.

Results

Measuring the fraction of the total pool of vesicles that participates in recycling

The main goal of this work was to quantify the fraction of the synaptic vesicle (SV) pool located at presynaptic terminals that participates in recycling during prolonged AP firing. We developed a protocol using pHluorin as an optical tracer to visualize and quantify both the total SV pool and the fraction of it that can be engaged to recycle. We have explicitly assumed that synaptic vesicles are the only spH-containing organelles in synaptic terminals (see Discussion). Figure 1a shows confocal images of a rat hippocampal neuron in culture transfected with spH, depicting GFP fluorescence intensity in a pseudo-color scale. The fluorescence of this pH-sensitive GFP fused to the lumenal aspect of VAMP-2 is largely quenched at the resting lumenal pH (5.5), and undergoes an
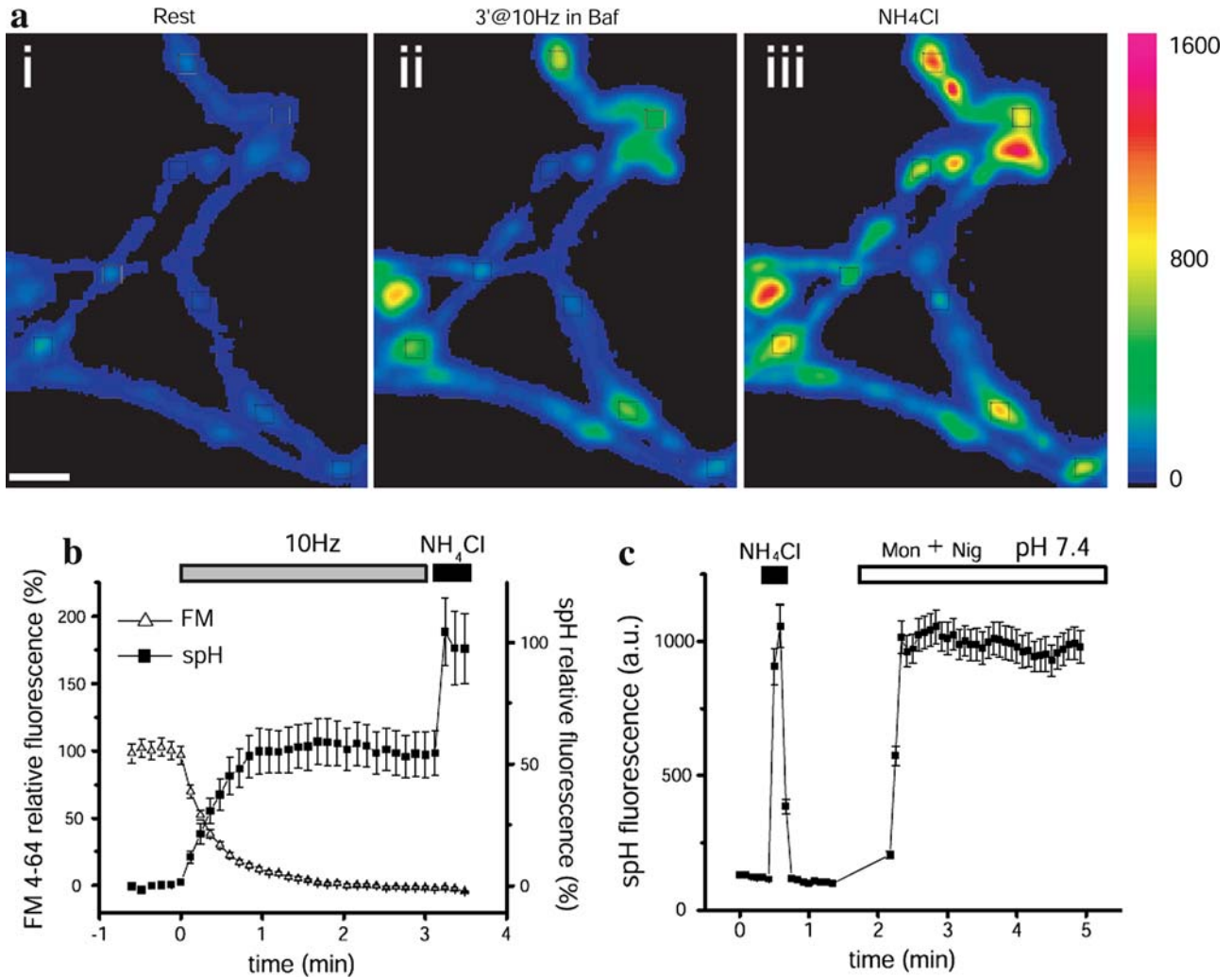
Fig. 1. Quantitative estimates of recycling and resting vesicle pool fractions in live cells. (a) Pseudo-color images of a representative axonal branch from a spH-transfected hippocampal neuron, during rest (i), after field stimulation with 1800 APs in the presence of 0.5 μM Bafilomycin (Baf) (ii), and during a subsequent application of NH_4Cl (50 mM, pH 7.4) to equilibrate synaptic-vesicle lumen pH to 7.4 (iii). Black boxes represent examples of areas selected for analysis. Only a subset is shown for clarity. Scale bar, 3 μm. Vertical pseudo-color scale shows spH fluorescence intensity values (arbitrary units, a.u.). (b) Representative experiment showing the protocol used to visualize the fraction of the total pool of synaptic vesicles participating in recycling. (Solid squares) Average spH fluorescence response of one spH-transfected neuron ($n = 49$ boutons) to 1800 APs (10 Hz, gray bar) normalized to the fluorescence level obtained during a subsequent NH_4Cl exposure (black bar). Baf (0.5 μM) was present throughout the experiment to prevent reacidification of vesicles after endocytosis (alkaline trapping); therefore any increase in spH fluorescence during stimulation reflects new vesicles undergoing exocytosis. The increase in fluorescence reached a steady-state level after ~600 AP, indicating the time required to turn over the recycling pool of vesicles. NH_4Cl application produced a further increase in fluorescence, reflecting the equilibration of resting vesicles to pH 7.4. FM 4-64 unloading (open triangles) was simultaneously recorded in the same set of boutons, which were preloaded with dye in a previous round of stimulation. The time to reach 50% recycling pool turnover was identical based on either spH or FM 4-64 responses (~180 AP). (c) NH_4Cl effectively "unmasks" the total pool of synaptic vesicles present at axon terminals. The spH fluorescence level reached with NH_4Cl (black bar) is the same as that reached when the pH of the vesicles was equilibrated to the extracellular pH (7.4) by applying 40 μM Monensin and 10 μM Nigericin (Mon + Nig, white bar), which allow neutral exchange of cations for protons across cell membranes.

~20-fold increase in its fluorescence when the pH shifts to 7.4 upon exocytosis (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). The fluorescence observed at rest (panel i in Fig. 1a) largely reflects VAMP-2 on the plasma membrane surface. Superfusion with NH_4Cl (50 mM) equilibrates the SV lumen to a pH of 7.4 (Miesenbock et al., 1998), thus unmasking clusters of vesicles located in axon terminals and allowing the visualization of individual boutons as puncta of fluorescence (panel iii). These puncta were chosen as regions of analysis in subsequent studies (square boxes in Fig. 1a, see section "Experimental procedures"). Synaptic vesicles are normally re-acidified after endocytosis by a V-type ATPase whose action can be blocked by the reversible cell-permeant inhibitor bafilomycin (baf). Measurements of spH fluorescence during AP stimulation in baf provide a direct measure of exocytosis, independent of any endocytic event, since vesicles become trapped in the alkaline state during recycling (alkaline trapping). Bafilomycin does not significantly perturb exocytosis or endocytosis in this system (Cousin and Nicholls, 1997; Sankaranarayanan and Ryan, 2001; Zhou et al., 2000; Li et al., 2005). To visualize the pool of vesicles that can be engaged to recycle, neurons were field stimulated with 1800 APs at 10 Hz (which far exceeds the stimulus required to turn over the entire recycling pool of SVs, see below) in the presence of baf (panel ii). The size of the vesicle pool that participates in recycling can be estimated for each bouton by subtracting the fluorescence during rest from that obtained after the stimulus (ΔF_{1800AP}, i.e., panel ii − panel i). A similar subtraction can be done to the fluorescence in NH_4Cl to get the size of the total vesicle pool (ΔF_{NH_4Cl}, i.e., panel iii − panel i). Finally, the fraction of the total pool of vesicles recycling can be calculated for each bouton as $\Delta F_{1800AP}/\Delta F_{NH_4Cl}$. Figure 1b shows the spH fluorescence increase during stimulation in the presence of baf (alkaline trapping) and a subsequent application of NH_4Cl, plotted as a function of time. A steady-state level is reached after ~600 APs, reflecting the stimulation required to turn over the entire recycling pool (Fig. 1b, solid squares). This complete turnover was confirmed by simultaneously measuring FM 4-64 destaining from the same set of boutons (Fig. 1b, open triangles), which were preloaded with the dye in a previous round of stimulation. FM 4-64 destaining kinetics agreed quantitatively very well with the kinetics of

alkaline-trapped spH fluorescence increase (time to half FM signal decay = 18 s, time to half spH signal increase = 20 s), further supporting the idea that this methodology provides a robust measure of AP-driven synaptic vesicle pool turnover. In general, this steady-state level in spH fluorescence was no different if neurons were stimulated at a frequency of 20 Hz and it was reached within 600–1200 APs, in agreement with previous reports (Ryan and Smith, 1995). To confirm that NH_4Cl (50 mM, pH 7.4) effectively equilibrates the lumen of synaptic vesicles with the extracellular pH, the peak fluorescence level reached in NH_4Cl was compared with that reached when a combination of ionophores that allow free exchange of cations for protons across cell membranes was applied. Figure 1c shows such an experiment. Monensin (40 μM) and nigericin (10 μM) were perfused in a Ca^{2+} and Na^+-free (replaced with K^+) saline to prevent vesicle fusion and allow pH equilibration across all membranous compartments (Lee and Epel, 1983; Lemasters et al., 1995). spH reached the same fluorescence level in both conditions (peak mon + nig/peak NH_4Cl = 0.96 ± 0.1, Mean ± SD of 6 independent experiments), indicating that pH inside SV-lumen is effectively equilibrated with the extracellular pH during NH_4Cl exposure. Taken together, these experiments indicate that spH fluorescence values reached during prolonged stimulation in baf, as compared to that reached under NH_4Cl, provides a good estimate of the fraction of the total pool of synaptic vesicles that can be engaged in recycling as well as the fraction that remains acidic and hence refractory to recycling.

The fraction of recycling vesicles varies from synapse to synapse and neuron to neuron

The distribution of recycling vesicle fractions in a population of 47 boutons arising from an individual neuron is presented in Fig. 2. For each bouton, the spH ΔF during both alkaline trapping and NH_4Cl application is depicted in Fig. 2a. Displaying the fraction recycling as a function of the entire vesicle pool shows that that terminals with larger vesicle pools do not necessarily have larger recycling fractions (Fig. 2b, but see also below and Fig. 3d). Figure 2c shows the frequency distribution of the fraction recycling for the 47 boutons analyzed. The percentage of vesicles recycling for this neuron varied from 0% to 100% for different boutons, with a mean of 54% with a standard deviation of 27%.
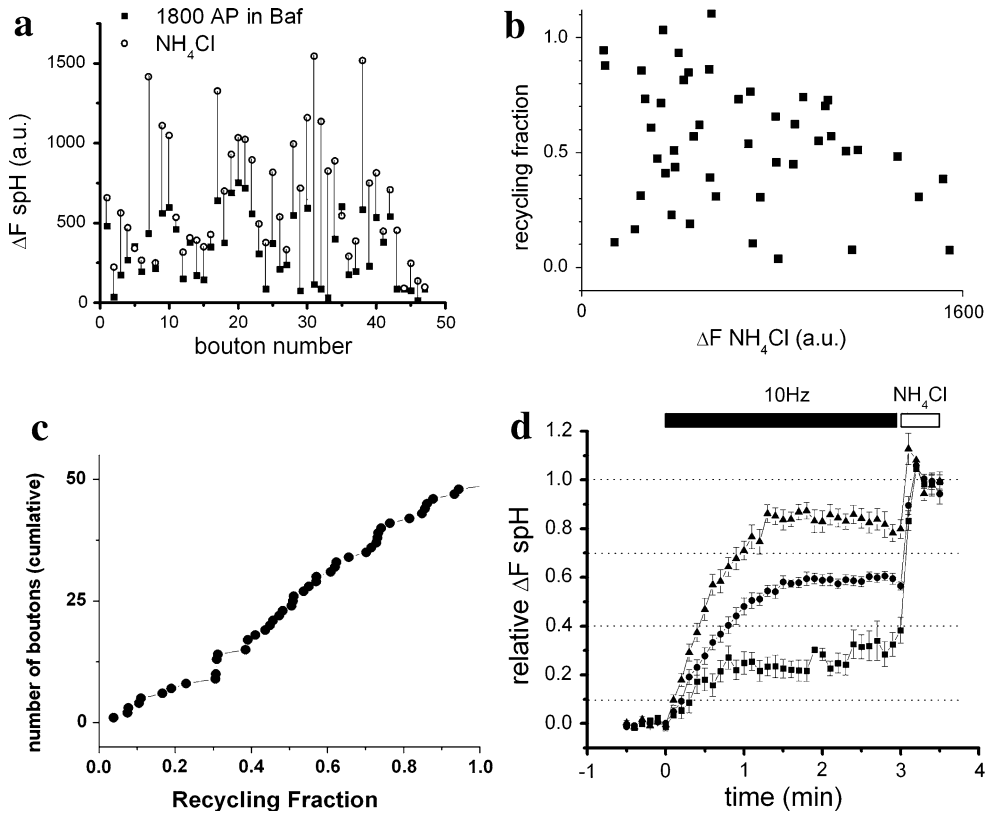
Fig. 2. Individual bouton analysis of the recycling vesicle pool fraction. The study of 47 boutons corresponding to one neuron (same as that presented in Fig. 1a) is shown. (a) The increase in spH fluorescence (with respect to the baseline at rest, ΔF spH) after 1800 APs in baf (solid squares) and after NH_4Cl perfusion (open circles) is shown for each bouton. The length of the line linking the symbols represents the size of the resting pool of vesicles. $\Delta F_{1800AP}/\Delta F_{NH_4Cl}$ was calculated to get the fraction recycling in (b) and (c). (b) The fraction of vesicles recycling is plotted as a function of the total vesicle pool size as reflected in the fluorescence increase in the presence of NH_4Cl. (c) Cumulative frequency histogram of the fraction of the total pool of vesicles that participates in recycling during prolonged stimulation (fraction recycling). The fraction recycling varied from 0 to 1 with an average value of 0.54 ± 0.04. (d) The fraction recycling does not appear to be related to the efficiency of the fusion machinery. Boutons were pooled in three groups according to the fraction of vesicles recycling: 0.1–0.4 (solid squares, $n = 11$); 0.4–0.7 (solid circles, $n = 17$); and 0.7–1 (solid triangles, $n = 14$). The average normalized fluorescence for each group is plotted as a function of time (dotted line delimits the three groups). The kinetics of exocytosis during stimulation (3 min at 10 Hz in baf, gray bar) is similar in the three groups ($\tau_{0.1-0.4} = 32$ s, $\tau_{0.4-0.7} = 37$ s, $\tau_{0.7-1} = 32$ s).

There were no obvious differences in the kinetics of vesicle pool mobilization and fusion among boutons that made use of a small, a medium, or a large fraction of its total pool to release neurotransmitter (0.1–0.4, 0.4–0.7, and 0.7–1 fraction groups, respectively), suggesting that the fraction recycling is independent of the efficiency of the fusion machinery (Fig. 2d). We found the average value to be $50 \pm 3\%$ across 18 cells (Fig. 3a, b). Using vGlut-pHluorin (vGpH) or synaptotagmin I-pHluorin (tagmin-pH) as reporters in identical experiments, we found that the average recycling pool size was very similar (Fig. 3a, $60.1 \pm 9.9\%$, vGpH, $n = 5$;

$51.0 \pm 10\%$ tagmin-pH, $n = 4$ cells). Analysis of recycling fractions recycling over many cells shows that in addition to varying across boutons the mean recycling fraction also varies from neuron to neuron (Fig. 3b). The average fraction recycling was 22% and 67% for the neurons presenting the smallest and the highest values, respectively (cells number 14 and 11, respectively, see Fig. 3b). Similar ranges were obtained for vGpH and tagmin-pH. We also assessed whether the fraction recycling varied with age of cultures. It was recently reported that the amount of FM 1-43 uptake is twofold higher in neuronal cultures of 15 days versus 12 days
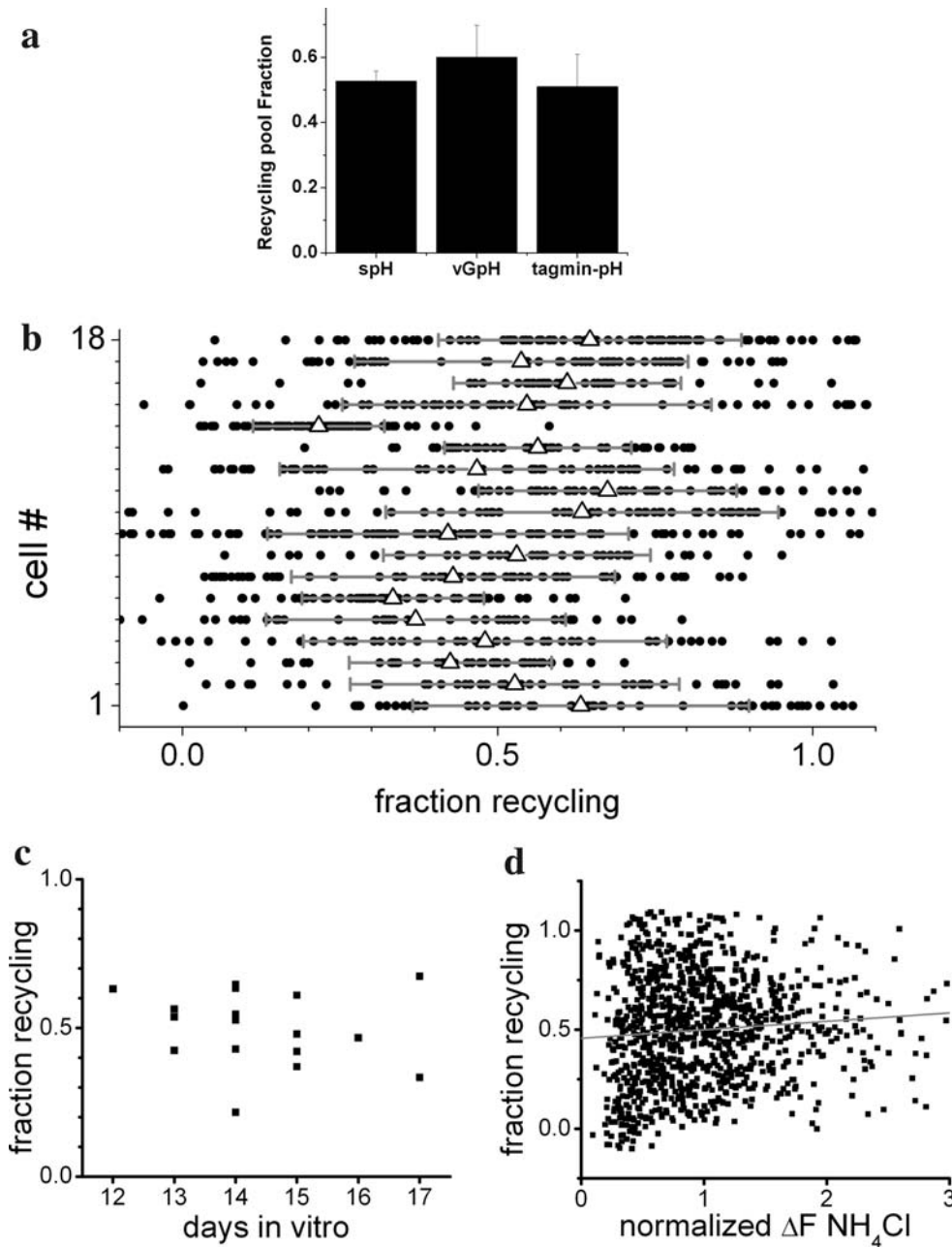
Fig. 3. The recycling vesicle fraction varies across different cells and synapses but represents on average 50% of the total pool. (a) Measurements of average fraction of recycling vesicles for spH ($n = 18$), synaptotagmin-pHluorin (tagmin-pH, $n = 4$), and vGlut1-pHluorin (vGpH, $n = 5$) are indistinguishable ($P = 0.05$ ANOVA). (b) Distribution of vesicle pool recycling fraction across different boutons in 18 cells from independent cultures. Each solid symbol represents a single measurement at a single bouton. The average recycling fraction and its standard deviation for each cell is shown as open triangles and gray bars, respectively ($n = 36$–96 boutons in each cell). (c) The average fraction recycling for each experiment is plotted as a function of the age *in vitro* of the individual culture. The fraction recycling did not correlate with age in culture for the range of ages analyzed (12–17 days *in vitro*). (d) Boutons containing larger vesicle pools have a slight tendency to have higher recycling fractions. The increase in spH fluorescence in response to NH_4Cl application (ΔF_{NH_4Cl}) gives an estimate of the size of the total vesicle pool. To compare across different neurons, this value was normalized to the average ΔF_{NH_4Cl} in each independent experiment. The gray line shows a linear fit to the data ($R = 0.09$; $P < 0.01$).

in vitro (Renger et al., 2001) suggesting that the number of vesicles recycling increases during synapse maturation. Contrary to what we expected, we found no increase in the fraction recycling in more mature cultures (Fig. 3c). However, it should be noted that if the total pool of vesicles increased proportionally to the recycling pool, then this fraction would not be affected. We also tested the hypothesis that boutons containing larger vesicle pools are able to engage a larger fraction of the pool in recycling. There is a very small ($R = 0.09$) but significant ($P < 0.01$) tendency of larger pools to recycle a larger fraction of its vesicles (Fig. 3d). Taken together, these experiments indicate that the fraction of the total pool of vesicles that recycles varies across synapses (0–100%) and across neurons (~20–70%), that it is not correlated with the age of the culture and that terminals with more vesicles are likely to recycle a larger fraction of its vesicles.

The fraction of vesicles recycling varies little in the time scale of hours

The heterogeneity in the fraction of vesicles recycling across boutons from a single cell (Fig. 3) may indicate that, at the single bouton level, the fraction of vesicles recycling could be modulated over time. To test this hypothesis, we measured the change in the fraction recycling at single boutons over a period of 3 h. Figure 4a shows that the average recycling fraction of all boutons remains stable over 3 h. Interestingly, some boutons showed large variations in the fraction recycling (Fig. 4b, black bars). However, these represented only 19% of all boutons and included increasing and decreasing fraction of recycling vesicles (Fig. 4b). Thus, although a few boutons present large variation in the fraction recycling over time, the overall fraction across all boutons appeared to remain stable over a period of hours.

Resting and recycling vesicles are interchanged across boutons

The stability in the recycling and resting vesicle pools (Fig. 4) may indicate that vesicles remain stably bound at their terminals. Alternatively this may reflect a steady state behavior, with a constant flux of vesicles into and out of these pools, interchanging across boutons or even between recycling and resting pools within boutons while maintaining a constant relative abundance. To test these possibilities, we developed an approach to photobleach the fluorescence of the entire pool of vesicles (i.e., recycling and resting) in individual boutons and examined recovery of fluorescence over time of each of the pools (Fig. 5). We reasoned that photobleaching the spH fluorescence in the presence of NH_4Cl at an individual bouton would irreversibly mark vesicles in both recycling and resting pools. A typical bleaching event is shown in Fig. 5. Prior to photobleaching the boutons show a robust NH_4Cl response reflecting the alkalinization of both the recycling and resting vesicles. Following NH_4Cl washout and a return to baseline fluorescence, the recycling pool can be mobilized to fuse with the plasma membrane with a 30 s 20 Hz stimulus (Fig. 5a). Individual boutons were then photobleached during a second NH_4Cl challenge. Five minutes after this bleaching episode, the NH_4Cl response at the bleached bouton was eliminated indicating both recycling and resting pools were bleached. A second round of AP stimulation gave rise to only a minimal signal, likely corresponding to diffusional spillover from adjacent nonbleached boutons (Sankaranarayanan and Ryan, 2000; Li and Murthy, 2001; Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006). Nearby boutons in the field retained both their NH_4Cl signal as well as the stimulus-driven recycling pool signal. Repeated querying of fluorescence with NH_4Cl and AP firing at different times after bleaching revealed that the fluorescence of both pools gradually returned to original values reaching near-completion within ~3 h ($87 \pm 7\%$ of total pool, and $81 \pm 11\%$ recycling fluorescence recovered, $n = 3$ boutons) (Fig. 5b–d). A final stimulation in the presence of bafilomycin followed by NH_4Cl confirmed that both recycling and resting vesicles are likely under dynamic exchange with neighboring boutons (Fig. 5e).

Discussion

We used a novel strategy to determine what fraction of vesicle pools in living synaptic terminals participates in vesicle recycling during prolonged stimulation. We relied upon measurements of genetically encoded tracers of synaptic vesicles, pHluorin-tagged synaptic vesicle proteins, to determine what portion of labeled vesicle pools can engage in recycling. In resting terminals, spH
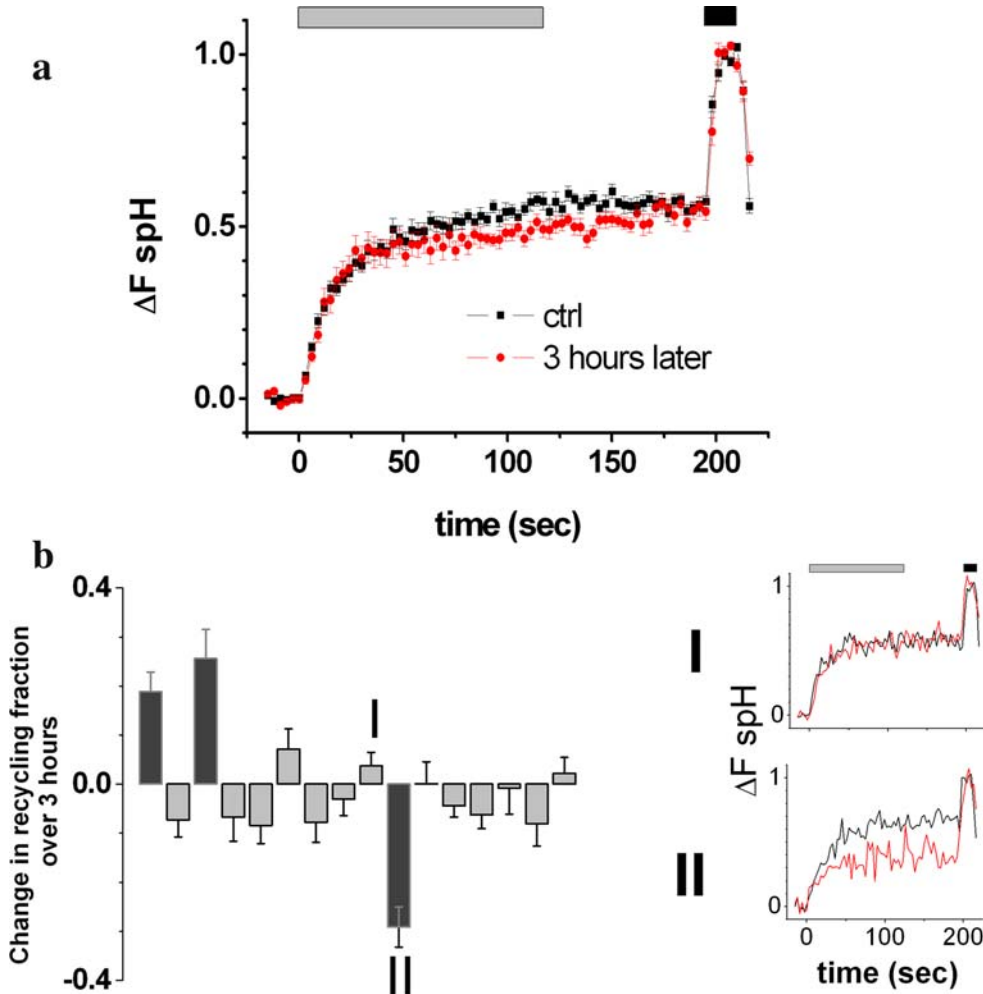
Fig. 4. The fraction of vesicles recycling is stable over a 3-h time period. (a) The average fraction of vesicles recycling measured in two consecutive trials with an interval of 3 h indicates that recycling and resting vesicles proportions are stable over time. Recycling fractions are 53 ± 2% and 47 ± 2% in the first and second trial, respectively (n = 16 boutons). (b) The majority of boutons showed little variation (< 10%) in the recycling fraction (13 boutons, dark gray bars). A few presented a larger variation (between 15% and 30%, n = 3 boutons, light gray bars). The error bars are the standard deviation of the steady-state fluorescence after all recycling vesicles were trapped in the alkaline state during the stimulus (2–6%). The inset depicts the example of individual boutons presenting a 4% increase (I) and 29% decrease (II) in their recycling fraction.

fluorescence is quenched at the low vesicular pH (~5.5). During recycling in the presence of bafilo-mycin, a blocker of the vesicular proton pump responsible for vesicle reacidification, vesicles become trapped in the alkaline (pH 7.4) and thus fluorescent state. If all labeled vesicles in the pool recycle, then the fluorescence should reach a value that would be equivalent to direct alkalization of vesicles to pH 7.4 (via either NH_4Cl or protono-phores). Any pHluorin-tagged protein that fails to

recycle will remain acidic and maintain a low fluorescence value.

The robustness of our estimates of synaptic vesicle pool recycling fractions with pHluorin-tagged proteins relies on two main assumptions. First that the proteins we examined, VAMP-2, vGlut-1, and Synaptotagmin I, and their pHluorin-tagged variants, reside exclusively in synaptic vesicles at synaptic terminals. An alternate possi-bility is that some of the reporters may be targeted

Fig. 5. Recycling and resting vesicle pools interchange across boutons. After assessing the size of the total vesicle pool with NH_4Cl and estimating its recycling pool with a train of stimulation (a), all vesicles in a single bouton where bleached (red arrow in right panel pictures, red trace in left plots). (b–d) The same protocol applied 5 min (b), 30 min (c), and 160 min (d) after bleaching indicates that nonbleached vesicles reached the originally bleached bouton and distribute in both recycling and resting pools. (e) After 3 h, nonbleached vesicles account for 91% of originally bleached ones (NH_4Cl, compare with a); of these, 51% are capable of recycling (stimuli in Baf). The response to the same series of trials is also shown for a neighboring, nonbleached, bouton (a–e, white arrow in right panel pictures, black traces in left plots). Scale bar 5 μm.
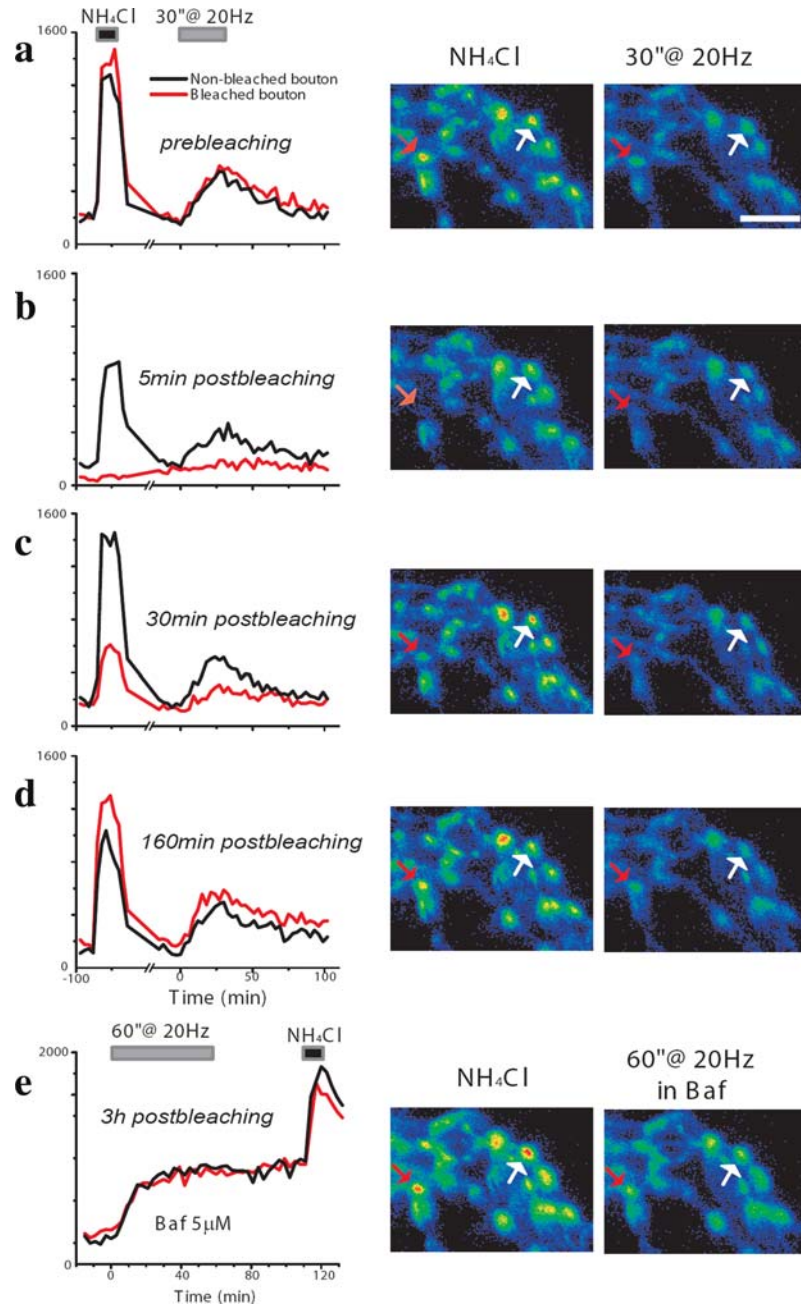
to endosomes as has been suggested for VAMP-2 (West et al., 1997). We feel this is unlikely since presynaptic spH appears to maintain a resting pH similar to that of synaptic vesicles, not endosomes (Miesenbock et al., 1998; Sankaranarayanan et al., 2000; Mukherjee et al., 1997), although the definition of an endosome could potentially be expanded to include the resting synaptic vesicle proteins we see here. The second assumption is that pHluorin-tagged proteins are randomly distributed between recycling and resting vesicle pools. At present, it is very difficult to explicitly test this hypothesis.

However, even if the expression of pHluorin-tagged SV proteins is biased toward one pool or another, the basic observation that a large fraction of the vesicles seem to be held in a state of "deep" reserve or are simply not competent for recycling will not change. We recently demonstrated that the expression of both spH (Fernandez-Alfonso et al., 2006) and vGpH (Balaji and Ryan, 2007) results in only 1–2 copies of the pHluorin-tagged protein per vesicle, much lower than the number of endogenous proteins likely to be present (Takamori et al., 2006). Thus, it seems unlikely that the appearance of these reporters in a resting compartment results from miss-targeting. Similarly, the fact that the most abundant synaptic vesicle proteins display similar partitioning argues that this is likely a bona-fide property of synaptic vesicles. Since we are measuring recycling vesicles relative to the total pool, it should be noted that, for example, a "silent" bouton does not necessarily mean a low fraction of vesicles recycling (i.e., a bouton that has a few vesicles recycling, although silent, will have a large fraction recycling if the total pool number is also very small; see Fig. 2a).

Our results are in reasonable agreement with other approaches for examining recycling vesicles based on photoconversion of FM 1-43 and electron microscopy (Harata et al., 2001). However, FM photoconversion experiments do not distinguish whether nonstained vesicles are the ones that fail to recycle or recycle in a manner that excludes dye labeling (i.e., kiss and run fusion). Since our method relies only on the exchange of protons within vesicles with the extracellular medium to track exocytosis, we conclude that resting vesicles are indeed the ones that do not fuse with the plasma membrane. Our approach also allows analyzing synaptic vesicle recycling in a vast number of synapses simultaneously, in living specimens, and in real time. We believe that the methods introduced in this work should add substantially in determining how various modulators of presynaptic function regulate recycling pool sizes.

Our photobleaching experiments indicate that both recycling and resting vesicle pools appear to exchange between synaptic boutons. Even at intermediate time points in the photobleaching recovery, the relative size of the recycling and resting pools (as judged by the peak of the NH_4Cl compared to the peak of the stimulus response) appears constant. This suggests that the two pools are always closely linked, perhaps even constantly interchanging within a given synapse over these time scales. Recently, it was demonstrated that FM 1-43-labeled and thus by definition recycling vesicles, exchange between boutons (Darcy et al., 2006). Our results complement those studies and demonstrate that this sharing of vesicles extends to nonrecycling vesicles as well. Here our experiments do not distinguish between possible recovery by local interchange with a recycling pool (which itself can recover by surface diffusional exchange of spH or the slower intracellular exchange) or long range mobility of resting vesicles. It is interesting to point out that the time scale of recovery we see is considerably slower than the time scale of movement of recycling vesicle between boutons observed by Darcy et al. (2006).

In Drosophila neuromuscular junctions, two spatially segregated pools of vesicles have been characterized; one located in the periphery and the other in the central region of presynaptic terminals (Kuromi and Kidokoro, 1998). Interestingly, the central one was shown to participate in recycling only after high-frequency stimulation or activation of protein kinase A (Kuromi and Kidokoro, 2000). Results using an approach very similar to ours with pHluorin and alkaline trapping has also been used in drosophila, which confirmed the existence of a synaptic vesicle pool that could only successfully be mobilized following endocytic block in the shibire mutant at nonpermissive temperatures (Poskanzer and Davis, 2004), which had previously been observed using other methods (Delgado et al., 2000). It seems that there exists a pool of vesicles in a "deep reserve" state that can be switched to participate in recycling and is conserved across species for regulating synaptic transmission. It is important to note, however, that we did not find any obvious spatial distinction of the two pools in our experiments (compare panels ii and iii in Fig. 1a). Although it is true that our system does not provide high enough resolution to detect fine spatial distributions, electron micrographs of FM photoconversion experiments suggest that recycling and resting vesicles are distributed at random in mammalian CNS (Harata et al., 2001).

In conclusion, we show that a varying fraction of synaptic vesicles remain in a resting state (i.e., do not participate in recycling) during prolonged stimulation at CNS synapses. Although in a resting state, it is tempting to consider that part of this pool could be tapped to undergo recycling, that would imply that synapses have a large reserve capacity

that might be used to modulate presynaptic function. It is also tempting to speculate that this resting pool might not only serve to regulate synaptic efficacy in individual boutons, but also be exchanged between synapses under some conditions. At present we do not know what physiological signal transduction pathways might normally control the balance of recycling and resting pools; however, the methods used here should prove useful in exploring how and when this "untapped" synaptic resource is utilized.

Methods

Cell culture and experimental conditions
Hippocampal CA3-CA1 regions were dissected from 2- to 4-day-old Sprague-Dawley rats, dissociated, and plated onto poly-ornithine-coated glass as previously described (Ryan, 1999). Cultures were maintained at 37°C in a 95% air, 5% CO_2 humidified incubator, and culture media was replaced every week. In spH experiments, calcium phosphate-mediated gene transfer was used to transfect 6- to 8-day-old cultures as previously described (Sankaranarayanan et al., 2000) with super-ecliptic spH, vGpH, or Tagim-pH. Super-ecliptic pHluorin is a modified version of the original ecliptic pHluorin with enhanced optical properties but an identical pK_a (~7.1) for protonation (Sankaranarayanan et al., 2000). Cells were used 12–17 days after plating (6–12 days after transfection), and the coverslips were mounted in a rapid switching, laminar-flow perfusion and stimulation chamber on the stage of a custom-built laser scanning confocal microscope. The total volume of the chamber was ~75 μl and was perfused at a rate of 1–1.5 ml/min. Action potentials were evoked by passing 1 ms current pulses, yielding fields of ~10 V/cm through the chamber via Platinum–Iridium electrodes. Unless otherwise noted cells were continuously perfused at room temperature (~24°C) in a saline solution containing in mM: 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES (buffered to pH 7.4), 30 glucose, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Research Biochemicals, Natick, MA) and 50 μM D,L-2-amino-5-phosphonovaleric acid (AP5; Research Biochemicals). When external $CaCl_2$ was varied, the total divalent ion concentration was kept constant by simultaneously changing $MgCl_2$. NaCl and CaCl$_2$ were replaced with KCl (keeping the total ion concentration fixed) during application of Monensin (40 μM, CalBiochem) and Nigericin (10 μM, Molecular Probes), two carboxylic ionophores that together allow the exchange of cations for protons across cell membranes (both drugs were stored frozen as 10 mM stock solutions in MeOH). When NH_4Cl (50 mM) was added to the saline, NaCl was reduced to 70 mM. Bafilomycin A1 (Baf; CalBiochem, San Diego, CA) was stored frozen as 0.2 mM aliquots (in DMSO) and diluted to a final concentration of 0.5 μM prior to spH experiments. In Fig. 1b, FM 4-64 (Molecular Probes, Eugene, OR) were used at a final concentration of 15 μM and vesicles were labeled by electrical field stimulation for 30 s at 20 Hz in the presence of the dye in normal saline, followed by an additional 60 s of dye exposure to ensure labeling of all recycling membrane during endocytosis. Unless otherwise noted all chemicals were obtained from Sigma (St. Louis, MO).

Optical measurements, microscopy, and analysis
Laser-scanning fluorescence images were acquired using a custom-built laser-scanning microscope through a 40× 1.3 numerical aperture Zeiss Fluar objective (Oberkochen, Germany). Specimens were illuminated with ~1.5 μW of the 488 nm line of an argon ion laser that was rapidly shuttered during all nondata acquiring periods using acousto-optic or electro-optic modulation. Time course of fluorescence response of spH and FM dyes was measured from time-lapse images taken every 3–10 s. SpH fluorescence emission was collected using a 500 nm long pass filter (except for combined FM 4-64 and spH experiments, where a 498–538 nm band pass filter was used for spH); FM 4-64 emission was collected using a 650 nm long pass filter. Quantitative measurements of fluorescence intensity at individual boutons were obtained by averaging 4 × 4 area pixels (0.4 × 0.4 μm^2) corresponding to the center of mass of 8 × 8-pixels positioned over individual puncta selected by hand from images (Fig. 1a shows a subset of areas selected). Further criteria for including a fluorescent puncta as a synaptic bouton in our analysis included requiring a minimum of a 1.5-fold increase in fluorescence upon alkalization with NH_4Cl compared to that at rest ($[F_{NH_4Cl} - F_{rest}]/F_{rest}$). Fluorescence values for baseline, prolonged stimulation

and NH$_4$Cl were obtained by averaging 2–5 images at rest, after reaching a steady-state level during stimulation (10 or 20 Hz), and during NH$_4$Cl exposure, respectively. The noise in our estimates of fraction recycling was calculated from fluorescence difference of the average of the first three images and the average of the following three images taken prior to stimulation (six images were taken before stimulation), relative to the average change in fluorescence in NH$_4$Cl (i.e., $[F_{1-3} - F_{4-6}]/\Delta F_{NH_4Cl}$). The standard deviation of the estimated noise was 0.11 ($n = 148$ boutons), therefore the fraction recycling was binned in 0.1 intervals. Four out of 22 neurons, which had high basal rates of fluorescence increase on bafilomycin application, were not included in the final analysis, since we could not distinguish with confidence whether the rise in fluorescence was due to spontaneous alkalization of vesicles or spontaneous vesicle fusions (Sankaranarayanan and Ryan, 2001). Data points that presented a fraction recycling of less than -0.1 or more than 1.1 probably represented slight shifts of boutons from focal plane and were not included in our analysis (55 out of 1,085 boutons). ΔF for each bouton was always normalized to ΔF_{NH_4Cl} before any further analysis. In Fig. 5, bleaching was achieved by scanning the laser beam over a region of interest encompassing a single bouton (~10 times more power and scanning rates than for regular imaging) in the presence of NH$_4$Cl for ~30 s. Errors are shown as standard error of mean except where stated.

Acknowledgments

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