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An Isolated Pool of Vesicles Recycles at Rest and Drives Spontaneous Neurotransmission

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Summary

Spontaneous synaptic vesicle fusion is a common property of all synapses. To trace the origin of spontaneously fused vesicles in hippocampal synapses, we tagged vesicles with fluorescent styryl dyes, antibodies against synaptotagmin-1, or horseradish peroxidase. We could show that synaptic vesicles recycle at rest, and after spontaneous exo-endocytosis, they populate a reluctantly releasable pool of limited size. Interestingly, vesicles in this spontaneously labeled pool were more likely to re-fuse spontaneously compared to vesicles labeled with activity. We found that blocking vesicle refilling at rest selectively depleted neurotransmitter from spontaneously fusing vesicles without significantly altering evoked transmission. Furthermore, in the absence of the vesicle SNARE protein synaptobrevin (VAMP), activity-dependent and spontaneously recycling vesicles could mix, suggesting a role for synaptobrevin in the separation of the two pools. Taken together these results suggest that spontaneously recycling vesicles and activity-dependent recycling vesicles originate from distinct pools with limited cross-talk with each other.

Introduction

All synapses manifest spontaneous neurotransmitter release in the absence of presynaptic action potentials (Katz, 1969). Over the last five decades, several studies have examined this form of vesicle fusion to elucidate the mechanisms of neurotransmitter release. In most cases, these low-probability release events correspond to a single quantum of neurotransmitter that presumably originates from fusion of a single synaptic vesicle (Frerking et al., 1997). Beyond providing great insight to the mechanisms of synaptic transmission and formulation of the quantal hypothesis of synaptic transmission (Del Castillo and Katz, 1954; Katz, 1969), these spontaneous release events may be required for signaling leading to maturation and stability of synaptic networks (McKinney et al., 1999; Verhage et al., 2000), inhibition of local dendritic protein synthesis (Sutton et al., 2004), or may even drive action potential firing in cells with high input resistance (Carter and Regehr, 2002; for review, see Otsu and Murphy, 2003). In contrast to the highly regulated and precisely timed nature of evoked neurotransmitter release, spontaneous synaptic vesicle fusion can only be loosely regulated by extracellular calcium, fluctuations in intracellular calcium, and neuromodulators (Angleson and Betz, 2001; Dittman and Regehr, 1996; Llano et al., 2000). This dichotomy led to a debate on the mechanism and location of spontaneous fusion (Colmeus et al., 1982; Deitcher et al., 1998; Van der Kloot, 1996). Recent studies have shown that spontaneous fusion is coupled to endocytosis (Ryan et al., 1997; Murthy and Stevens, 1999; Sun et al., 2002), suggesting the presence of a recycling pathway that operates at rest. These spontaneous release events are generally assumed to be due to low-probability fusion of docked synaptic vesicles that are already primed for release (Murthy and Stevens, 1999). However, this premise has never been directly tested. To elucidate the mechanisms that lead to spontaneous synaptic vesicle fusion, we employed electrophysiological and optical imaging techniques that allowed us to track the synaptic vesicles' history of use. Using these approaches, we found that distinct vesicle pools with limited cross-talk sustain spontaneous versus activity-dependent synaptic vesicle recycling.

Results

Synaptic Vesicles Recycle at Rest and Preserve Their Molecular Identity during Recycling

To visualize spontaneous vesicle trafficking at presynaptic terminals, we incubated mature hippocampal cultures with AM1-44, which is a fixable version of the styryl dye FM1-43, together with a polyclonal antibody generated against the lumenal domain of the synaptic vesicle protein synaptotagmin 1 (syt1). The incubation was carried out for 15 min in the presence of tetrodotoxin (TTX) to block action potential firing. AM1-44 indiscriminately labels endocytosing membranes, whereas the antibody against the lumenal domain of syt1 can specifically label endocytosing synaptic vesicles under the same condition (Malgaroli et al., 1995; Matteoli et al., 1992). Figure 1 shows that the distribution of fluorescent AM1-44 puncta after spontaneous dye uptake was similar to the labeling with syt1 antibody visualized by a fluorescent secondary antibody after permeabilization. AM1-44 and syt1 staining patterns avoided dendrites and cell bodies but marked presynaptic terminals. Fluorescent intensities of the two labels showed significant colocalization, suggesting that the AM1-44 signal originates from synaptic vesicles as identified by their syt1 immunoreactivity (Figures 1A–1E). We also compared spontaneous AM1-44 labeling to the activity-dependent uptake of syt1 antibody in the same synapse. Both signals were again colocalized as measured by the correlation of pixel fluorescence intensities (Figures 1F–1J). In contrast to the spontaneous uptake of both probes, which resulted in similar fluorescence intensities (Figure 1E), activity-dependent labeling with

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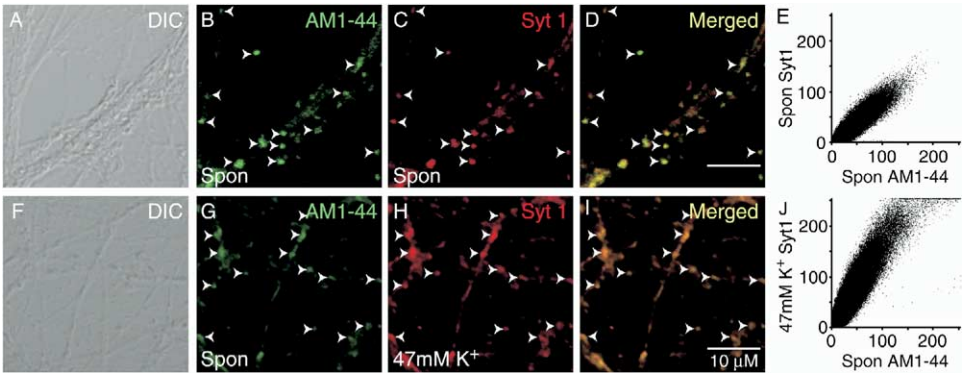

Figure 1. Visualization of Spontaneous Endocytosis in Hippocampal Cultures

(A–D) Fluorescent images of synapses loaded by spontaneous AM1-44 uptake (B) and spontaneous uptake of synaptotagmin 1 (syt1) lumenal domain antibody (C) show colocalization (D), suggesting that the fixable FM1-43 analog (AM1-44) is taken up into synapses by spontaneously recycling synaptic vesicles.

(E) Cytofluorogram (joint distribution of pixel intensity values from the two fluorescence detection channels) showing colocalization of spontaneous AM1-44 and syt1 antibody uptake.

(F–I) Fluorescent images of synapses loaded by spontaneous AM1-44 uptake (G) and 47 mM K^+ (activity) induced uptake of synaptotagmin 1 (syt1) luminal domain antibody (H) show colocalization (I), suggesting that the fixable FM dye (AM1-44) is taken up by the same synapses that show activity-dependent vesicle recycling.

(J) Cytofluorogram showing colocalization of spontaneous AM-144 and activity-dependent syt1 antibody uptake. The leftward skew is consistent with a larger pool of activity-dependent recycling vesicles than spontaneously recycling vesicles (see Figure 4E).

syt1 resulted in brighter fluorescence compared to spontaneous AM1-44 uptake (Figures 1I and 1J).

We next tested whether spontaneously endocytosed synaptic vesicles recycle and become reavailable for fusion and thus take up a second probe. To test this possibility, we exposed cultures to the antibody against the lumenal domain of syt1 for 15 min in the presence of TTX. After wash out of the primary antibody (>15 min), we incubated cultures with the fluorescently conjugated secondary antibody (in the presence of TTX), which can only recognize the primary antibody once it is exposed to the extracellular medium (Figure 2A). This experiment resulted in significant punctate immunolabeling of hippocampal cultures without permeabilization (Figure 2B). When we introduced the secondary antibody through permeabilization after the spontaneous uptake of the primary antibody, the amount and the pattern of fluorescence labeling was comparable to the one seen before when the secondary antibody was introduced by uptake without permeabilization (Figures 2D, 2E, and 2H). To check the amount of syt1 epitope available on the surface membrane at any given time

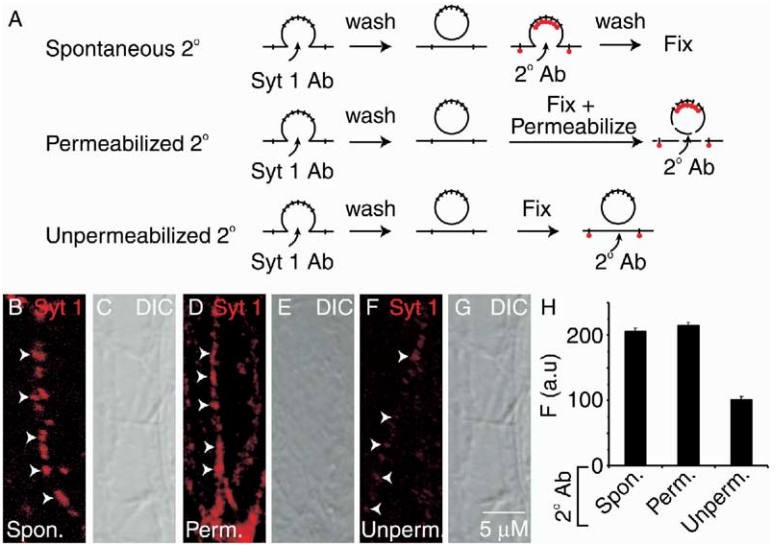

Figure 2. Spontaneous Recycling of Synaptic Vesicle Protein Synaptotagmin 1

(A) Experimental protocol. Synapses were loaded spontaneously with synaptotagmin 1 luminal domain antibody. After wash out of the primary antibodies, secondary antibodies were either loaded spontaneously (spontaneous 2°) or delivered after fixation. In the case of "permeabilized 2°" experiments, secondary antibody was delivered after permeabilization with saponin. For "unpermeabilized 2°" experiments, synapses were fixed and secondary antibodies loaded without permeabilization to check for surface epitopes.

(B–G) Synapses that were labeled with the secondary antibody spontaneously (B and C) or after fixation and permeabilization (D and E) show strong punctuate fluorescence, while synapses labeled with the secondary antibody after fixation without permeabilization only show weak fluorescence (F and G), suggesting that the fluorescent labeling is primarily due to uptake of secondary antibody into spontaneously recycling vesicles and not entirely due to residual surface antibody or fluorescence background.

(H) Quantification of fluorescence intensity of puncta in the three loading paradigms (n = 150 synapses for each condition, 10 cells, 15 synapses per cell).

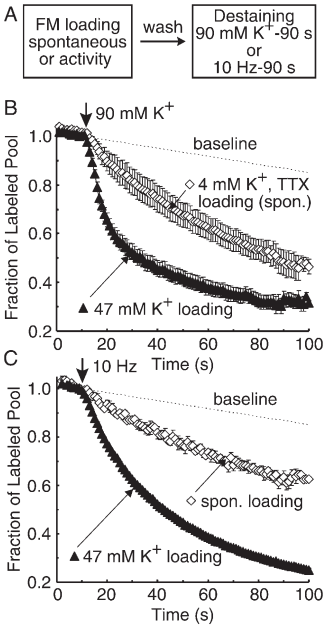

Figure 3. Spontaneously Endocytosed Vesicles Preferentially Populate a Reluctant/Reserve Pool

(A) Experimental protocol.

(B) Spontaneously loaded synapses (N = 10 [# of experiments], (495) [# of synapses]) show slow monophasic destaining kinetics in response to 90 mM K^+ application. In contrast, the same synapses loaded maximally by high potassium stimulation display rapid biphasic destaining.

(C) Application of action potentials at 10 Hz to hippocampal cultures results in slower destaining of synapses loaded in the presence of TTX (N = 3 (119)) compared to synapses labeled with 47 mM K^+ stimulation.

during spontaneous vesicle recycling, we labeled cultures with the secondary antibody after fixation but without permeabilization (Figures 2A, 2F, and 2G). This maneuver resulted in substantially diminished fluorescence labeling, which can be partly due to nonspecific reactivity of the secondary antibody. This finding suggests that most of the fluorescence in earlier experiments originated from internalized syt1 (Figure 2H). Taken together, the re-availability of the primary syt1 antibody for interaction with the fluorescent secondary after a 15 min wash period indicates that synaptic vesicles are spontaneously recycled with minimal loss of their molecular identity.

Spontaneously Endocytosed Vesicles Preferentially Populate a Reluctant/Reserve Pool

If some synaptic vesicles recycle at rest, then do these vesicles randomly mix with the activity-dependent recycling vesicles? To address this question, we exposed hippocampal cultures to the styryl dye FM2-10 for 10 min in the presence of TTX (Figure 3A). This allowed vesicles that exocytose spontaneously and subsequently endocytose to take up FM2-10. After dye washout, spontaneously loaded synapses emerged as fluorescent puncta (similar to AM1-44 puncta seen in Figure 1) that could subsequently be destained in response to stimulation. Surprisingly, application of 90 mM K^+/2 mM Ca^{2+} depolarizing solution to the spontaneously stained puncta resulted in slow monophasic destaining (Figure 3B). In contrast, when we labeled the same set of synapses with elevated potassium (47 mM K^+) stimulation after a 10 min rest period, they were labeled intensely and destained with the typical biphasic pattern generally observed in response to a 90 mM K^+ challenge (Klingauf et al., 1998) (Figure 3B). When we challenged spontaneously labeled synapses with 10 Hz field stimulation, this low-intensity stimulation also led to slower destaining compared to the destaining induced by 10 Hz stimulation of the same synapses labeled with 47 mM K^+ stimulation (Figure 3C). This result suggests that spontaneously endocytosed vesicles selectively populate a reserve pool from which they are only reluctantly available for release.

To examine this premise in depth, we investigated three additional parameters. First, we analyzed the rates of 90 mM K^+-induced dye loss from individual synapses labeled through spontaneous fusion or elevated potassium (47 mM K^+) stimulation (see Figure S1 available with the Supplemental Data available with this article online). Interestingly, this analysis revealed that approximately 10% (out of 295) of synapses showed rapid destaining after spontaneous dye labeling, albeit still slower than dye release after activity-dependent labeling (see Figure S1 legend). This finding is in agreement with previous observations (Prange and Murphy, 1999). However, such rapid destaining patterns had random occurrence within the synapse population since there was no correlation between the rate of destaining and initial fluorescence levels nor with the amount of fluorescence sequestered within the synapses determined after background subtraction ($R^2 < 0.05$) (Figure S1). Second, we tested whether the rate of destaining was affected by the duration of dye wash out after staining. When we rapidly washed out FM dye within 2 min (instead of the usual 10 min) using fast perfusion (10 ml/min) and applied 90 mM K^+ stimulation immediately after dye washout, spontaneously labeled synapses still destained slower than synapses labeled with activity (Figure S2), suggesting that the 10 min time delay for dye wash did not mask a fast component of release for spontaneously labeled synapses. Finally, we examined whether synapses labeled through spontaneous recycling could release dye in response to hypertonic sucrose stimulation. Brief hypertonic sucrose application releases vesicles from the readily releasable pool (Rosenmund and Stevens, 1996). Therefore, if spontaneously labeled vesicles randomly mixed within the total activity-dependent recycling pool, then some of these vesicles should be resident within the readily releasable pool and thus could be released by hypertonic sucrose application. When we perfused hyperosmolar solution (+0.5 M sucrose) onto spontaneously labeled synapses, they did not release appreciable dye (Figure S3). In contrast, synapses labeled with activity released more than 20% of the dye in response to the same stimulation, similar to previous observations (Pyle et al., 2000; Mozhayeva et al., 2002). These three observations reinforce the hypothesis that spontaneously fused vesicles once endocytosed do not mix with the total recycling pool and evade populating the readily releasable pool.

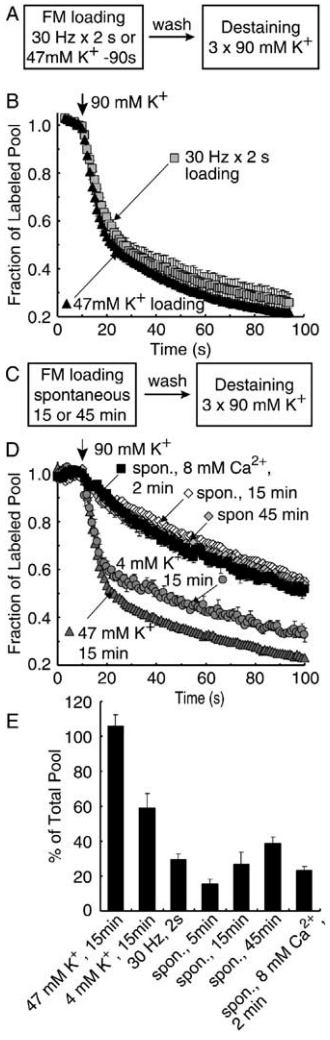

Figure 4. The Kinetics of Destaining Is Strictly Dependent on the Presence or Absence of Stimulation during the Loading Phase but Not to the Duration of Dye Labeling or the Number of Vesicles Labeled

(A) Experimental protocol. Synapses were loaded with FM dye using either a 30 Hz × 2 s AP stimulation or 47 mM K^+ stimulation. Following a 10 min washout period, synapses were imaged during the application of 90 mM K^+ solution.

(B) The kinetics of vesicle mobilization is similar for synapses loaded by activity irrespective of the size of the fluorescently labeled pool.

(C) Experimental protocol.

(D) Synapses loaded by spontaneous exo-endocytosis of vesicles in 4 mM K^+/2 mM Ca^{2+} (in TTX) for 15 (or 45) min (N = 7 (691) and 7 (571), respectively) or in 4 mM K^+/8 mM Ca^{2+} (in TTX) solution for 2 min (N = 3 (239)) all show slow monophasic release kinetics during 90 mM K^+ induced destaining. In contrast, synapses loaded by activity induced by 47 mM K^+ for 15 min (N = 3 (318)) or by the network activity existing in the neuronal circuits in the culture (4 mM K^+ 15 min; N = 3 (305)) show biphasic kinetics similar to synapses loaded with high potassium for 90 s.

(E) Normalized fluorescence values (ΔF) for the different loading paradigms described above. While 30 Hz × 2 s stimulation loads a pool size comparable to the synapses loaded by the various spontaneous loading protocols, the kinetics of vesicle mobilization after 30 Hz × 2 s is faster (D). Changing the duration of spontaneous loading or the concentration of Ca^{2+} does not affect the size of the labeled pool. Removing of TTX from the solution to allow network activity increases the size of the pool loaded (E), but also changes the distribution of loaded vesicles (D). Prolonged application of 47 mM K^+ for 15 min does not significantly increase the size of labeling over that labeled during 90 s. Decreasing the duration of spontaneous dye loading from 15 min to 5 min, however, significantly decreased the amount of dye labeling (0.15 ± 0.03 for 5 min versus 0.27 ± 0.07 for 15 min, $p < 0.05$).

Vesicles Labeled with Minimal Activity Randomly Mix within the Total Recycling Pool

Comparison of the amount of dye loaded after spontaneous and 47 mM K^+ staining protocols revealed that during 10 min spontaneous activity we could label only about 25% of the total recycling pool stained using the 47 mM K^+ challenge. To test whether the extent of dye loading or the number of dye-loaded vesicles is a determinant of subsequent destaining kinetics, we labeled synapses with 60 action potentials applied within 2 s (30 Hz × 2 s) (Figure 4A). This stimulation stained approximately 30% of the total vesicle pool, and, in contrast to the behavior of spontaneously labeled vesicles, application of 90 mM K^+/2 mM Ca^{2+} solution resulted in biphasic destaining. The kinetics of destaining was identical to that seen after labeling of the total recycling pool with 47 mM K^+ stimulation (Figure 4B). This result suggests that vesicles labeled with 30 Hz × 2 s stimulation randomly mix within the total recycling pool. In the case of spontaneously exo-endocytosed vesicles, however, equal mixing of endocytosed vesicles within the recycling pool does not occur during the 10 min wash out period after dye uptake. The comparable magnitude of dye labeling after 30 Hz × 2 s stimulation and spontaneous labeling indicates that this observation is not simply due to a low signal-to-noise ratio at low fluorescence intensities but is an outcome of the presence or absence of stimulation during dye uptake.

The Size of the Spontaneously Endocytosed Vesicle Pool Is Limited and Independent of the Duration of Dye Labeling or External Ca^{2+} Concentration

We next tested whether longer duration incubation with FM dye would increase dye labeling and give more time for vesicle mixing and thus change the destaining kinetics of spontaneously labeled vesicles. Incubating hippocampal cultures with FM2-10 for 45 min in the presence of TTX did not significantly increase dye labeling from the level seen after 15 min incubation (Figure 4E). Furthermore, in response to 90 mM K^+ stimulation the destaining kinetics of these spontaneously labeled synapses was still monophasic and slow compared to the mobilization of vesicles after activity-dependent labeling (Figures 4C and 4D). However, reducing the duration of spontaneous labeling to 5 min significantly decreased dye uptake compared to 15 min labeling ($p < 0.05$), indicating that 5 min is not sufficient for mobilization of the total pool of spontaneously recycling vesicles (Figure 4E).

To further examine the relationship between the conditions for dye uptake and ensuing high potassium induced dye release, we elevated extracellular Ca^{2+} from 2 mM to 8 mM to increase spontaneous fusion rate (3.02 ± 0.74-fold increase measured electrophysiologi-

cally [n = 5]). In 8 mM Ca^{2+}, a 2 min incubation with FM dye labeled up to 25% of the total recycling pool. Vesicles stained in this manner still destained slowly when exposed to a 90 mM K^+ challenge. In contrast, incubation of synapses in 47 mM K^+ solution for 15 min or 4 mM K^+ solution for 15 min in the absence of TTX (to allow network activity) resulted in a significantly higher level of labeling with these synapses displaying typical biphasic destaining kinetics during 90 mM K^+ stimulation (Figures 4D and 4E). Taken together, these results show that the kinetics of destaining was strictly dependent on the presence or absence of stimulation during the loading phase but not to the duration of dye labeling or the number of vesicles labeled. In addition, irrespective of extracellular Ca^{2+} levels or the duration of stimulation, spontaneous dye uptake labeled a limited pool size, less than one-third of the total recycling pool, which was only reluctantly releasable during sustained stimulation (Figure 4E). Our analysis also showed that the size of the spontaneously labeled vesicle pool was correlated with the size of the total recycling pool labeled with high K^+ stimulation in a given synapse (R^2 = 0.67 ± 0.05, n = 8). This result indicates that spontaneous and activity-dependent dye uptake labels the same presynaptic terminal as suggested in an earlier study by Prange and Murphy (1999). However, this correlation was not as tight as the one we normally observe between 30 Hz × 2 s labeling and the total recycling pool size (R^2 = 0.84 ± 0.03, n = 6). This is consistent with the argument that spontaneous dye uptake and 30 Hz × 2 s labeling originate from distinct pools.

Vesicles in the Spontaneously Labeled Pool Are More Likely to Re-Fuse Spontaneously

What is the relative tendency of spontaneously endocytosed vesicles to re-fuse spontaneously? To address this question, we labeled synaptic terminals either through spontaneous dye uptake or activity-dependent stimulation. Then, after dye wash out, we monitored the rate of spontaneous fluorescence decay for 20 min (Figure 5A). In these experiments, to minimize fluorescence loss due to photobleaching, we acquired images using brief exposures at 30 s time intervals. Surprisingly, spontaneously stained synapses showed more pronounced spontaneous destaining compared to fluorescence destaining after activity-dependent loading with 30 Hz × 2 s or high K^+ stimulation (Figure 5B). Despite the difference of staining levels reached after high K^+ stimulation versus the brief burst of action potentials (Figure 5B, inset), both staining levels lost less than 25% of their initial amplitudes within 20 min (Figure 5B). In contrast, synapses labeled with spontaneous dye uptake showed accelerated destaining with the loss of 45% of the initial level within the same time frame. Thus the acceleration in the rate of spontaneous dye loss was not dependent on the number of labeled vesicles but was rather a consequence of the absence of stimulation during dye uptake. This result suggests that spontaneously endocytosed vesicles were more likely to be reused spontaneously.

We next compared the rate of spontaneous dye loss in the presence of 2 mM or 8 mM Ca^{2+} after spontaneous dye uptake. In 8 mM Ca^{2+}, spontaneous dye loss was significantly faster in accordance with the earlier observation that elevated extracellular Ca^{2+} concentration increases frequency of spontaneous fusion (Figure 5C). We also tested whether prolonged loading (10 min) in the presence of background network activity (0.83 ± 0.15 Hz, n = 3) altered the rate of subsequent spontaneous dye loss. Vesicles labeled in this way were mostly endocytosed in response to network activity since the amount of dye uptake is at least 2-fold larger than spontaneous dye uptake (in TTX) for the same duration. These vesicles endocytosed during network activity were also slow in their ability to fuse spontaneously compared to vesicles that were labeled spontaneously (black diamonds, Figure 5C). These findings also confirm the specificity of the spontaneous dye loss paradigm we used here to monitor spontaneous vesicle recycling.

Spontaneous Recycling Can Be Fit with a Single-Pool Model

Previous studies have estimated the rate of spontaneous vesicle fusion per synapse to be on the order of one vesicle per 90 s (Geppert et al., 1994; Murthy and Stevens, 1999). This estimate is faster than the rate we observed for dye loss from spontaneously labeled vesicles in the absence of stimulation (Figure 5B). A simplified kinetic model allowed us to resolve this discrepancy with the assumption that spontaneously endocytosed vesicles rapidly mix with other vesicles in the same pool within seconds (~2 s) (Figure 5D). This assumption is in line with a recent finding by Sun et al. (2002) who demonstrated that spontaneously fused vesicles can be endocytosed quite rapidly within 100 ms, as well as findings of Pyle et al. (2000) that endocytosed synaptic vesicles rapidly mix with vesicles from the same pool. Incorporation of these parameters in a simple recycling scheme can accurately describe our data. In the absence of this recycling step, dye release is expected to decline by more than 60% within 20 min (Figures 5B and 5D). Furthermore, this kinetic model, by a 2-fold increase in the rate of spontaneous exocytosis "α," could also successfully account for the increased rate of dye loss in the presence of 8 mM Ca^{2+} in the extracellular medium (Figures 5C and 5D). However, it is possible that increased extracellular Ca^{2+} may also affect other parameters such as the rate of recycling "β" (Figure 5D).

Blocking Vesicle Refilling at Rest Selectively Depletes Neurotransmitter from Spontaneously Fusing Vesicles

Optical experiments we described so far suggest that a discrete pool of spontaneously recycling vesicles give rise to the electrophysiologically detected spontaneous fusion events (miniature synaptic currents, or "minis"). To test this prediction, we designed the following experiments in which we tagged spontaneously recycling vesicles by blocking neurotransmitter refilling after endocytosis at rest (Zhou et al., 2000) (Figure 6A). Recordings obtained after 10 min incubation of hippocampal cultures with the vacuolar ATPase inhibitor folimycin in the presence of TTX demonstrated a 6-fold reduction of mini frequency (Figures 6B, 6C, and 6E). In contrast,

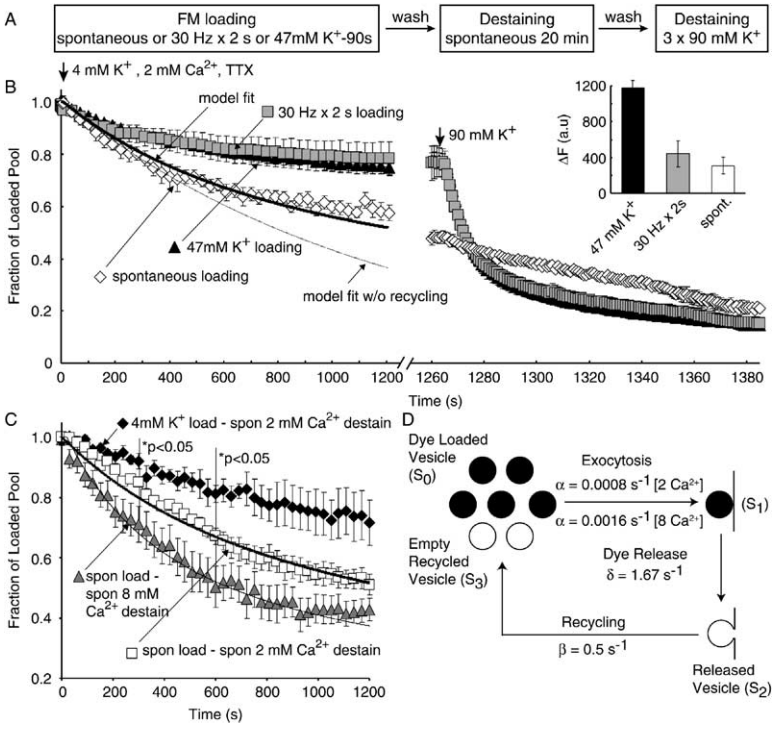

Figure 5. Vesicles in the Spontaneously Labeled Pool Are More Likely to Re-Fuse Spontaneously

(A) Experimental Protocol.

(B) Spontaneously loaded vesicles preferentially re-exocytose spontaneously. Approximately 45% of the spontaneously loaded pool had spontaneously exocytosed after 20 min (N = 9 (571), less than 25% of the 47 mM K^+ or the 30 Hz × 2 s loaded pool (N = 6 (605) and 10 (983), respectively) had fused spontaneously within the same time frame ($p < 0.01$). (Inset) F values for different loading paradigms. Even though a larger pool of vesicles is loaded by 47 mM K^+ stimulation compared to spontaneous or 30 Hz × 2 s loading, the kinetics of spontaneous exocytosis from the activity-dependent pool is slower than the spontaneous pool (B).

(C) Spontaneous recycling is regulated by extracellular Ca^{2+} concentration. Spontaneously loaded vesicles showed faster exocytosis kinetics in the presence of 8 mM Ca^{2+} compared to 2 mM Ca^{2+}. While 33% of the spontaneously loaded pool had released in 10 min with 2 mM extracellular Ca^{2+}, 50% had released with 8 mM extracellular Ca^{2+} (N = 5 (557) and 7 (696), respectively, $p < 0.05$). Vesicles that were loaded with endogenous network activity present in the cultures for the same time as in the spontaneous loading paradigms (10 min) showed slower spontaneous release kinetics compared to spontaneously loaded vesicles (19% release by 10 min, N = 5 (539), $p < 0.05$), similar to activity loaded vesicles in Figure 5B.

(D) Spontaneous dye loss from the spontaneously labeled pool can be described with a simple model in which a fixed set of vesicles are released with a rate of one vesicle per 120 s (for 2 mM extracellular Ca^{2+}) and recycle with a rate of one vesicle per 2 s (bold line in [B] and [C]). In the presence of 8 mM extracellular Ca^{2+}, the data could be fit with an exocytosis rate of one vesicle/60 s, keeping the recycling rate constant (lower line in [C]).

this treatment only produced a 20% decrease in the amplitudes of minis (Figure 6F). Similarly, the decrease in the size of evoked synaptic current was only 25% after prolonged folimycin treatment (Figures 6D and 6G). These observations are consistent with the results of the earlier optical experiments demonstrating that spontaneously fusing vesicles originate from a distinct pool other than the ones that fuse in response to presynaptic action potentials. Therefore, blocking vesicle refilling at rest (in TTX) selectively depletes neurotransmitter from spontaneously recycling vesicles, since these vesicles would lose their neurotransmitter upon exo-endocytosis. In contrast, activity-dependent recycling vesicles are only affected by neurotransmitter leakage due to slow intravesicular alkalinization during folimycin treatment (Sankaranarayanan and Ryan, 2001). However, evoked transmission in folimycin-treated cultures showed a rapid use-dependent depression during repetitive stimulation, suggesting these vesicles also cannot be refilled with neurotransmitter once they recycle (data not shown).

In order to directly monitor the effect of folimycin, we recorded minis during folimycin application during the 10 min period. In these experiments, we detected a significant gradual drop in mini frequency, whereas the amplitude of individual events remained relatively constant (Figure S4). This finding is consistent with the premise that the decrease in mini frequency is due to "all or nothing" loss of electrical quantal responses resulting from the absence of neurotransmitter refilling during vesicle recycling. The decrease in mini frequency recorded during the 10 min folimycin perfusion was about 2-fold less than the decline seen in Figure 6. We believe this difference is due to the 5–10 min delay between the incubation with folimycin and the actual recordings shown in Figure 6.

Ultrastructural Identification of Spontaneously Recycling Synaptic Vesicles

To visualize the spontaneously recycling vesicles at the ultrastructural level, we quantified the uptake of horseradish peroxidase (HRP) using electron microscopy (Heuser and Reese, 1973). In these experiments, hippocampal cultures were treated with 90 mM K^+ for 120 s or were incubated with TTX for 15 min. HRP was present during the depolarization period or during the TTX treatment. In 90 mM K^+ treated synapses, the number of labeled vesicles per synapse per section was around ten (9.7 ± 1.2, n = 22, Figures 7A and 7B). In contrast, spontaneously labeled vesicles were few in number (4 ± 0.4, n = 22, per synapse/section, range = 1–8, median = 4, Figures 7A and 7B). Both activity-dependent vesicles and spontaneously labeled vesicles had similar normal morphology (Figure 7C), and all labeled vesicles appeared to be evenly distributed within the synaptic vesicle cluster. These observations complement previous experiments using antibodies against synato-

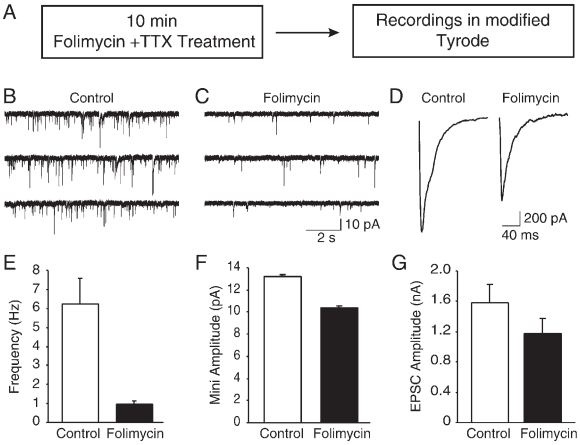

Figure 6. Blocking Vesicle Refilling at Rest Selectively Depletes Neurotransmitter from Spontaneously Fusing Vesicles

(A) Experimental Protocol.

(B and C) Sample traces of miniature postsynaptic currents from vehicle (DMSO)-treated (B) and folimycin-treated cultures (C).

(D) Sample evoked responses for vehicle-treated and folimycin-treated cultures.

(E) Summary graph showing 85% reduction in the frequency of spontaneous events (N = 7 and 8 for vehicle- and folimycin-treated cells, respectively; $p < 0.01$).

(F) Summary graph showing 21% reduction in amplitude of spontaneous events (N = 7 and 8 for vehicle- and folimycin-treated cells, respectively; $p < 0.01$).

(G) Summary graph showing 25% reduction in amplitude of evoked postsynaptic responses (N = 29 and 27 for vehicle- and folimycin-treated cells, respectively; $p = 0.19$).

tagmin-1 and FM dyes and suggest that the activity-dependent and spontaneously recycling vesicles have a molecular rather than an overt physical difference.

The Role of Synaptobrevin/VAMP in Spontaneous Synaptic Vesicle Recycling

To gain insight to the molecular mechanisms that underlie spontaneous synaptic vesicle recycling, we monitored spontaneous FM dye uptake and release in embryonic hippocampal cultures derived from synaptobrevin-2 (VAMP-2)-deficient mice. Synaptobrevin-2 is the major SNARE protein of synaptic vesicles and is required for fast Ca^{2+}-triggered synaptic vesicle exocytosis. The genetic deletion of synaptobrevin-2 partially abolishes fusion. Synaptobrevin-2-deficient (*syb2*$^{-/-}$) synapses showed a 6-fold reduction in spontaneous fusion frequency, and they still exhibited ~10% of wild-type (wt) release when stimulated by an application of hypertonic sucrose (Schoch et al., 2001). In contrast, the mutant synapses displayed <1% of wt release when stimulated by Ca^{2+} influx during an action potential. However, *syb2*$^{-/-}$ synapses were capable of vesicle recycling on a slow timescale in response to high potassium stimulation or repetitive action potential stimulation (Deák et al., 2004). We first tested littermate wt cultures for the validity of our earlier observations in postnatal rat cultures (Figure 8). Spontaneous dye loss after activity-dependent dye loading (47 mM K^+) was

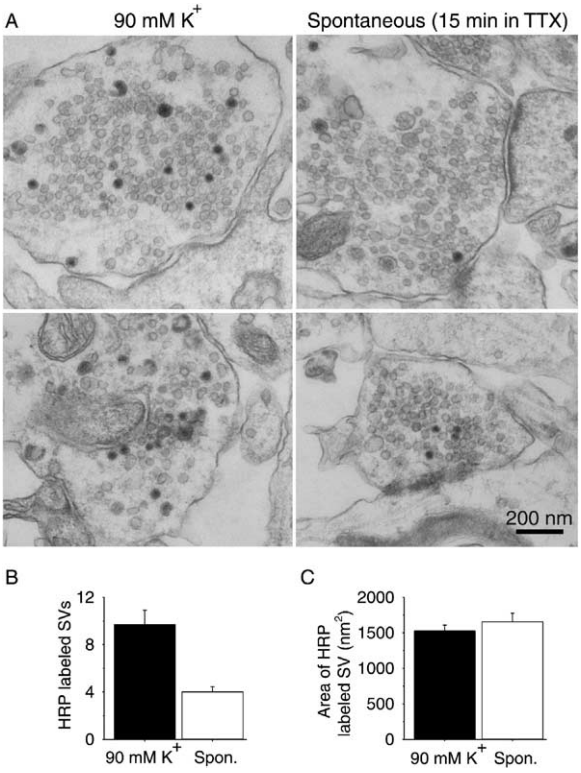

Figure 7. Electron Microscopy of Spontaneous Uptake of Horseradish Peroxidase into Synaptic Vesicles

(A) Representative synapses labeled with horseradish peroxidase (HRP) during activity-dependent (left panels) and spontaneous staining (right panels). For activity-dependent HRP labeling, hippocampal cultures were treated with 90 mM KCl containing solution for 120 s. HRP was present only during the stimulation. For spontaneous labeling, cultures were incubated with HRP in the presence TTX for 15 min.

(B) The number of HRP-positive synaptic vesicles (SVs) was higher in the case of activity-dependent loading compared to spontaneous HRP uptake ($n \geq 20$ for all cases).

(C) We did not detect a clear morphological or size difference, as depicted by labeled vesicle area, between the two vesicle populations.

significantly slower than dye loss from vesicles labeled spontaneously (Figure 8B). In the *syb2*$^{-/-}$ synapses, in agreement with previous electrophysiological experiments, the overall rate of spontaneous fusion was 3-fold slower than wt synapses. In addition, the amount of spontaneous dye labeling was 2-fold reduced compared to wt synapses. We attribute this apparent reduction in the difference between wt and *syb2*$^{-/-}$ synapses (compared to the earlier electrophysiological results) to the inherent bias toward selection of brighter puncta presumably corresponding to more active synapses during analysis of optical experiments. However, in striking contrast to wt synapses, the *syb2*$^{-/-}$ synapses showed similar rates of spontaneous dye loss after activity-dependent and spontaneous dye uptake. This observation suggested that, in the absence of synaptobrevin, activity-dependent and spontaneous vesicle pools mix randomly. Next, we tested this premise using 90 mM K^+ induced destaining. Here, as in rat cultures,

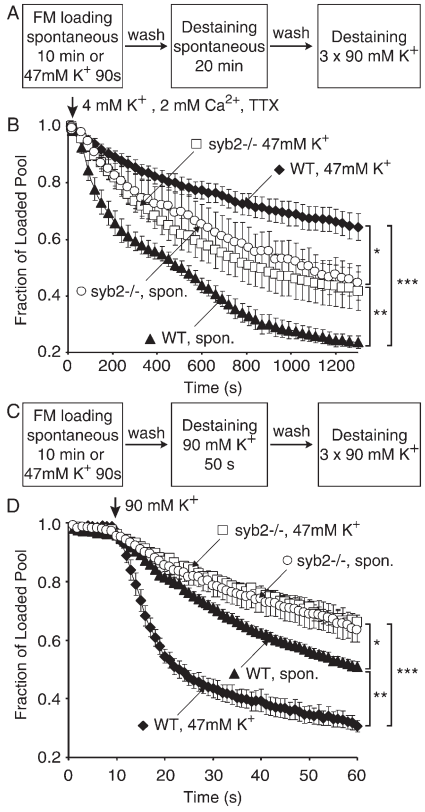

Figure 8. The Role of Synaptobrevin/VAMP in Spontaneous Synaptic Vesicle Recycling

(A) Experimental Protocol.

(B) Spontaneously loaded vesicles in cultures lacking synaptobrevin-2 (*syb2*$^{-/-}$) show slower spontaneous release kinetics compared to wild-type (wt) synapses (71% release in wt compared to 47% in *syb2*$^{-/-}$ after 15 min, N = 7 each, $p < 0.01$). Synaptic vesicles loaded with activity-dependent stimulation show slower spontaneous release in wt compared to *syb2*$^{-/-}$ cultures (30% release in wt compared to 52% in *syb2*$^{-/-}$, N = 7 each, $p < 0.05$). While spontaneously loaded vesicles in the wt embryonic mouse cultures (similar to rat cultures) preferentially re-released spontaneously compared to activity-loaded synapses ($p < 0.001$), surprisingly, spontaneous exocytosis of vesicles in *syb2*$^{-/-}$ synapses was independent of the presence of activity during dye uptake ($p > 0.6$).

(C) Experimental protocol.

(D) In wt cultures, spontaneously loaded vesicles showed slow activity-dependent mobilization compared to activity-loaded vesicles (similar to rat cultures). Synapses lacking synaptobrevin-2 showed similar activity-dependent dye release patterns irrespective of loading protocol. The kinetics of this release was significantly slower than wt synapses loaded either spontaneously ($p < 0.05$) or with activity ($p < 0.01$). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

spontaneously labeled vesicles in littermate wt synapses displayed slow activity-dependent destaining in response to 90 mM K^+. In contrast, vesicles labeled with activity displayed typical robust destaining. In *syb2*$^{-/-}$ synapses, vesicles labeled either with activity (47 mM K^+) or with spontaneous dye uptake destained slowly with similar rates. Taken together, these findings suggest that, in the absence of synaptobrevin-2, activity-dependent and spontaneously recycling vesicle pools mix, implicating the involvement of a synaptobrevin-mediated process in segregation of the two pools.

Discussion

The results of this study suggest that synaptic vesicles recycle at rest, which can be detected by uptake and reavailability of an antibody against the lumenal domain of synaptic vesicle protein synatotagmin-1 as well as internalization and release of styryl dyes. From these findings, we can reach three major conclusions on the nature of spontaneous vesicle recycling in hippocampal synapses. First, spontaneously endocytosed vesicles preferentially populate a reluctant/reserve pool, which has limited cross-talk with vesicles in the activity-dependent recycling pool. Second, the size of this spontaneously recycling vesicle pool is limited. Finally, vesicles in the spontaneously recycling pool are more likely to re-fuse spontaneously. The evidence that supports these results can be summarized as follows: using uptake and release of fluorescent styryl dye FM2-10, we could show that the kinetics of dye release strictly depends on the manner in which the dye was taken up into vesicles. Whenever we allowed activity (action potentials or high K^+) during dye uptake, the vesicles were equally mixed within the total recycling pool and showed similar rapid mobilization kinetics in response to sustained stimulation. In contrast, these activity-dependent recycling vesicles were not as readily available for release in the absence of stimulation. This situation was the opposite of what we observed with spontaneous dye uptake. Vesicles that took up dye during spontaneous exo-endocytosis were only reluctantly available for release during sustained stimulation, whereas they were swiftly mobilized in the absence of activity compared to activity-dependent recycling vesicles. Here, it is interesting to note that vesicles labeled in response to hypertonic sucrose stimulation can be mobilized with similar kinetics as activity-dependent recycling vesicles, suggesting that they equally mix within this pool (Mozhayeva et al., 2002; Pyle et al., 2000). This result is not surprising, since vesicles released by hypertonic sucrose stimulation originate from the readily releasable pool and therefore are more likely to mix with other activity-dependent recycling vesicles. Accordingly, we were unable to release a significant fraction of spontaneously labeled synaptic vesicles by hypertonic sucrose stimulation (Figure S3). These two observations are consistent with this study, demonstrating that most of the spontaneously fusing vesicles originate from a vesicle pool other than the readily releasable pool.

We could also verify the predictions of the optical experiments with an approach where we electrophysiologically tagged spontaneously recycling synaptic vesicles using a blocker of vacuolar ATPase. This maneuver selectively depleted neurotransmitter from spontaneously recycling vesicles and thus resulted in a 6-fold decrease in miniature postsynaptic current frequency. In contrast, the amplitudes of evoked events were minimally affected by this treatment, suggesting that the vesicles that give rise to evoked events were not recycled spontaneously.

These findings raise the question whether spontaneously recycling vesicles fuse at the active zone or at an ectopic site as suggested by some earlier studies in the neuromuscular junction (Colmeus et al., 1982; but see

Van der Kloot, 1996). The evidence presented in this study does not exclude either possibility. However, we should note that a recent study in the frog neuromuscular junction showed that the docked vesicle pool might not directly correspond to the readily releasable pool (Rizzoli and Betz, 2004). This finding increases the plausibility of the argument that both spontaneous and activity- dependent vesicles can fuse at the active zone despite their distinct origins.

The results presented here address a major question raised by earlier work on whether there are distinct vesicle trafficking pathways for spontaneous and evoked release in synapses. The only insight to this question so far has come from genetic studies which showed that spontaneous fusion rate was unchanged in the knockout of synaptotagmin 1 or complexins, proteins critical for evoked synchronous neurotransmitter release, suggesting lack of a Ca^{2+} triggering step for spontaneous fusion (Geppert et al., 1994; Reim et al., 2001). Loss of the active zone scaffolding protein RIM1α impairs vesicle priming, thus hypertonic sucrose-induced and Ca^{2+}-evoked transmission, but does not significantly alter spontaneous fusion rate (Calakos et al., 2004). In contrast, spontaneous fusion rate is significantly reduced after deletion of synaptobrevin-2/VAMP2 or completely abolished after genetic deletion of munc-18 or munc-13 isoforms (Schoch et al., 2001; Varoqueaux et al., 2002; Verhage et al., 2000). The selective role of proteins such as synaptotagmin 1, complexin, and RIM1α in evoked neurotransmitter release in contrast to the substantial role of synaptobrevin, munc-18, or munc-13 in both forms of vesicle trafficking is consistent with the premise that the two forms of release may originate from distinct recycling pathways. Interestingly, *Drosophila* neuromuscular junctions mutant in rab5, a small GTPase critical for vesicle trafficking through early endosomes, showed no differences in the frequency and amplitude of miniature excitatory junction potentials compared to wild-type junctions (Wucherpfennig et al., 2003). In contrast, evoked neurotransmitter release probability was significantly altered in these mutants, supporting the argument that both forms of release operate through distinct vesicle trafficking pathways. This premise is further supported by experiments performed by Koenig and Ikeda in the *Drosophila* neuromuscular junction where they monitored the recovery of evoked and spontaneous synaptic responses after *shibire*-induced vesicle depletion (Koenig and Ikeda, 1999). In this study, they observed that the active zone population of vesicles and evoked neurotransmitter release recovered in parallel within 30 s; in contrast, full recovery of spontaneous release took 10–15 min and required the recovery of the non-active zone population of vesicles.

In summary, these earlier results are consistent with our finding that spontaneous and activity-dependent vesicle recycling operate independently with limited cross-talk. This view may seem to contradict the notion that spontaneous release occurs through low-probability random fusion of primed vesicles in the absence of an external trigger (Murthy and Stevens, 1999; Prange and Murphy, 1999). However, it is important to note that in the current study we cannot fully exclude this possibility; we only postulate that the spontaneous fusion probability of activity-dependent vesicles is low, at the limit of our technical resolution. Therefore, some spontaneous fusion events may still arise from primed vesicles, albeit with a lower probability. The major caveat of our approach is that we only have an indirect way of assessing if the two vesicle populations are different. High-resolution ultrastructural analysis of vesicles after spontaneous and activity-dependent uptake of distinguishable probes would be the most direct way of testing whether the two sets of vesicles are indeed distinct. The limited electron microscopic analysis in this study did not reveal an obvious physical (or spatial) difference between the vesicle populations labeled with spontaneous versus activity-dependent uptake of HRP. Therefore, we propose that the functional segregation of the two sets of vesicles may be mediated by differences in the protein and/or lipid composition of the synaptic vesicles that make up the two pools. This premise is difficult to ascertain in the absence of direct biochemical evidence for synaptic vesicle heterogeneity. The finding that the antibodies to synaptotagmin-1 can readily label spontaneously recycling vesicles as well as the activity-dependent vesicles (Figures 1 and 2) argues against the absence or presence of synaptotagmin-1 as the underlying reason for this phenomenon. In contrast, our experiments in synaptobrevin-2-deficient synapses suggest a role for molecular interactions of synaptobrevin-2 or a synaptobrevin-2-dependent process (e.g., priming) in the segregation of the activity-dependent and spontaneous pools. The exact nature of the molecular diversity between the two vesicle pools and the mechanistic role of synaptobrevin-2 in this process remains to be identified. A divergence in the molecular composition of vesicles, as suggested by our findings, may also make differential regulation of these two recycling pathways a possibility. Such selective regulation may provide neural networks a means to distinguish between evoked and spontaneous synaptic activity.

Experimental Procedures

Cell Culture

Dissociated hippocampal cultures were prepared from 2- to 3-day-old Sprague-Dawley rat pups using previously described methods (Kavalali et al., 1999). Experiments were performed after 15–25 days in vitro, corresponding to a time period when synapses reach full maturity in culture (Mozhayeva et al., 2002).

For the analysis of synaptobrevin-deficient synapses, mice that are heterozygous mutant for synaptobrevin-2 (gift of Dr. T.C. Südhof) were set up for timed pregnancy. Hippocampal neurons from embryonic day 18 littermate mice were dissociated and cultured using previously described protocols (Schoch et al., 2001). These cultures were used after 15–25 days in vitro.

Immunocytochemistry

The cells were loaded with AM1-44 (Biotium Inc., Hayward, CA) in the presence of 1 μM tetrodotoxin (TTX, Calbiochem, La Jolla, CA) for 15 min. Polyclonal syt1 lumenal domain antibody (1:100, gift of Dr. T.C. Südhof) was either loaded in the presence of TTX for 15 min or in a 47 mM K^+ solution for 90 s. Secondary antibody (Alexa 594, 1:500, Molecular Probes, Eugene, OR) was either loaded spontaneously to live cells in the presence of TTX for 15 min or after fixation for 30 min with 4% paraformaldehyde in PBS containing 4 mM EGTA. In cases when permeabilization was performed, cells were first fixed and then incubated in 1X PBS containing 2% goat serum and 0.4% saponin (Sigma, St Louis, MO) for 1 hr before

addition of the secondary antibodies (1:500). The coverslips were then mounted onto frosted uncharged slides. Images were obtained with a Leica TCS confocal microscope, and the data were analyzed with the Leica confocal software.

Fluorescence Imaging

Modified Tyrode's solution used in all experiments contained 150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 2 mM $CaCl_2$ (pH 7.4, ~310 mOsm). High K^+ solutions contained equimolar substitution of KCl (90 mM) for NaCl. Synaptic boutons were loaded with FM2-10 (400 μM, Molecular Probes, Eugene, OR) or AM1-44 (16 μM, Biotium Inc, Hayward, CA) under conditions outlined in the text. Incubation for 90 s in 47 mM K^+ solution (Tyrode's solution with equimolar substitution of KCl [47 mM for NaCl]) gives maximal labeling of the recycling vesicle pool in a given synapse (Harata et al., 2001). Spontaneous loadings were performed in modified Tyrode's solution containing 1 μM TTX to inhibit action potentials induced by network activity inherent in the culture. Images were taken after 10 min washes in dye-free solution with nominal Ca^{2+} to minimize spontaneous dye loss. Destaining of hippocampal terminals with high-potassium challenge was achieved by direct perfusion of solutions onto the field of interest by gravity (2 ml/min). In a typical experiment, high potassium challenge was applied at least three times (for 90 s each separated by 60 s intervals) to release all of the dye trapped in presynaptic terminals. All staining and destaining protocols were performed in the presence of 10 μM CNQX and 50 μM AP-5 to prevent recurrent activity. Field stimulation was applied through parallel platinum electrodes immersed into the perfusion chamber delivering 30 mA, 1 ms pulses. In all experiments, we selected isolated boutons (~1 μm^2) for analysis and avoided apparent synaptic clusters. We did not observe a significant difference in background fluorescence levels between synapses loaded during brief periods of activity or prolonged periods of spontaneous vesicle release (275 ± 20 a.u. for spontaneously loaded synapses and 336 ± 50 a.u. for activity-dependent loaded synapses, $p = 0.29$). We did not observe any noticeable focus drift in the majority of experiments. Those experiments where focus drift occurred were easily identifiable by a coordinated jump in the destaining curves of all synapses and were discarded. Images were obtained by a cooled-intensified digital CCD camera (Roper Scientific, Trenton, NJ) during illumination (1 Hz, 40 ms) at 480 ± 20 nm (505 DCLP, 535 ± 25 BP) via an optical switch (Sutter Instruments, Novato, CA). Images were acquired and analyzed using Axon Imaging Workbench Software (Axon Instruments, Union City, CA). All statistical analyses were performed using Student's two-tailed t test using the number of coverslips as N unless stated otherwise. Experimental results are represented as mean ± SEM.

Electron Microscopy

Cells were either treated with 90 mM K^+ and HRP (10 mg/ml, Sigma) containing Tyrode's solution for 120 s or 4 mM K^+ Tyrode's solution containing HRP and TTX for 15 min. Coverslips were quickly rinsed and fixed for 30 min with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C. For 3,3′-diaminobenzidine (DAB) reaction, coverslips were incubated in Tris-Cl buffer (100 mM, pH = 7.4) containing DAB (0.1%) and H_2O_2 (0.02%) for 15 min. They were then rinsed twice in buffer and incubated in 1% OsO_4 for 30 min at room temperature. After rinsing with distilled water, specimens were stained en bloc with 2% aqueous uranyl acetate for 15 min, dehydrated in ethanol, and embedded in Poly/Bed 812 for 24 hr. Sections (60 nm) were post-stained with uranyl acetate and lead citrate and viewed with a JEOL 1200 EX transmission microscope.

Electrophysiology

Cultures were incubated in the modified Tyrode's solution containing 67 nM folimycin (Calbiochem, La Jolla, CA) dissolved in DMSO (Sigma, St Louis, MO) or vehicle alone, at 37°C for 10 min in the presence of 1 μM TTX. Following treatment, pyramidal cells were voltage clamped to −70 mV using whole-cell patch-clamp technique. Electrode solution contained 115 mM Cs-$MeSO_3$, 10 mM CsCl, 5 mM NaCl, 10 mM HEPES, 0.6 mM EGTA, 20 mM TEA.Cl, 4 mM Mg-ATP, 0.3 mM Na_2GTP, and 10 mM QX-314 (Sigma, St Louis, MO, pH 7.35, 300 mOsm). Data were acquired using an Axopatch 200B amplifier and Clampex 8.0 software (Axon Instruments, Union City, CA). Recordings were filtered at 2 kHz and sampled at 5 kHz. Spontaneous events were recorded in the presence of 1 μM TTX. For measuring evoked responses, electrical stimulation was delivered through parallel platinum electrodes in modified Tyrode's solution without CNQX or AP-5. All statistical comparisons were performed with two-tailed unpaired t test; values are given as mean ± SEM.

Modeling Spontaneous Synaptic Vesicle Recycling

The recycling of spontaneous vesicles was fit with a single-pool model with four states: s_0, dye-loaded vesicles; s_1, mobilized vesicles; s_2, empty vesicles; s_3, recycled and mixed empty vesicles; using rate constants α, rate of mobilization; δ, rate of dye loss; β, rate of recycling. The data in Figure 5 could be fit with $\alpha = 0.0008\ s^{-1}$ (~1 vesicle per 120 s for a pool of 15 vesicles), $\delta = 1.67\ s^{-1}$, $\beta = 0.5\ s^{-1}$ (Figures 5B, bold line, and 5C).

$$\frac{ds_0}{dt} = -\alpha \cdot s_0 \cdot \left(\frac{s_0}{s_0 + s_3}\right)$$

$$\frac{ds_1}{dt} = -\delta \cdot s_1 + \alpha \cdot \left(\frac{s_0}{s_0 + s_3}\right) \cdot s_0$$

$$\frac{ds_2}{dt} = -\beta \cdot s_2 + \delta \cdot s_1$$

$$\frac{ds_3}{dt} = \beta \cdot s_2$$

Changes in the rate of exocytosis caused dramatic shifts in the simulated destaining pattern (data not shown). The rate of recycling also altered the kinetics of dye loss due to the mixing parameter, although not as dramatically as changes to the exocytosis rate. The pool size used in the simulation shown is 15 vesicles; however, since the plot shows kinetics normalized to the total pool size, as is the case with the FM measurements, the absolute pool size (i.e., the number of vesicles used for the simulation) did not affect the normalized kinetics of destaining. Once vesicles in the simulation exocytose and release their dye, the empty vesicles were then equally mixed with the population of vesicles that remained labeled. Without this mixing, the kinetics of destaining was more rapid (Figure 5B).

Supplemental Data

Supplemental Data including four figures is available at http://www.neuron.org/cgi/content/full/45/4/563/DC1/.

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