nature neuroscience

A common origin of synaptic vesicles undergoing evoked and spontaneous fusion

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There is a longstanding controversy on the identity of synaptic vesicles undergoing spontaneous versus evoked release. A recent study, introducing a new genetic probe, suggested that spontaneous release is driven by a resting pool of synaptic vesicles refractory to stimulation. We found that cross-depletion of spontaneously or actively recycling synaptic vesicle pools occurred on stimulation in rat hippocampal neurons and identified the recycling pool as a major source of spontaneous release.

Neurotransmitter release is triggered by an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). However, spontaneous release of neurotransmitter at resting $[Ca^{2+}]_i$ is a common feature of all synapses. It gives rise to miniature postsynaptic potentials (minis), which were initially described to define the size of the quantal unit of synaptic transmission[1]. These action potential–independent minis were considered to arise from low-probability fusion of single synaptic vesicles (about one per 90 s)[2] that are docked and primed for release. Recent studies on the Ca^{2+} activation of vesicle fusion found that spontaneous transmitter release close to resting $[Ca^{2+}]_i$ is just a consequence of the properties of the molecular machinery that drives fusion[3].

In the ongoing controversial debate on the origin of spontaneous versus evoked release[4–8], it was recently proposed that the resting pool of synaptic vesicles, usually refractory to stimulation, is mobilized during spontaneous activity and is therefore responsible for minis[7]. The authors used a genetic probe termed biosyn (biotinylated synaptobrevin 2) to tag synaptic vesicles in hippocampal neurons on the basis of biotinylation of the synaptic vesicle protein synaptobrevin 2 (Syb2), which could then be labeled by fluorescent streptavidins when exposed to the surface; that is, during synaptic vesicle recycling. The major drawback of this technique, however, is that release can be monitored, but not the fate of labeled synaptic vesicles on a further round of stimulation; that is, which pool of synaptic vesicles they recycle. To resolve this 'one-shot experiment' problem, we refined the biosyn approach and coupled a new pH-sensitive cyanine dye, cypHer 5E[9], to unlabeled monovalent streptavidin[10], avoiding possible impairment because of cross-linking introduced by the tetravalent wild-type probe (**Supplementary Fig. 1**, **Supplementary Note 1** and **Supplementary Methods**). The fluorescence of the cypHer dye was maximal at intravesicular pH of 5.5 and was almost quenched at the slightly basic extracellular pH of 7.4 after synaptic vesicle exocytosis (**Fig. 1a**). Using cypHer-streptavidin, we were able to label synaptic vesicles in live hippocampal boutons expressing biosyn either by stimulation (900 action potentials at 20 Hz to mobilize the entire recycling pool) or by spontaneous activity (15 min at 37 °C in the presence of the sodium-channel inhibitor tetrodotoxin, TTX) (**Fig. 1b**). Neurons were then stimulated with 200 action potentials at 20 Hz to test the release competence of synaptic vesicles that had been stained either during evoked or spontaneous release (**Fig. 1c**). We found that the average cypHer fluorescence traces for both labeling conditions showed rapid decreases in fluorescence on stimulation with near-identical relative amplitudes, followed by a slow increase as a result of compensatory endocytosis. Thus, spontaneously and stimulation-labeled synaptic vesicle pools can be depleted by stimulation to the same degree, suggesting that there is one common synaptic vesicle pool.

We confirmed these results using live hippocampal boutons labeled with an exogenous cypHer-coupled antibody[11] (αSyt1-cypHer) against the luminal domain of the synaptic vesicle protein synaptotagmin1 (Syt1) following both protocols described above (**Fig. 1b**). Re-acidification of the intravesicular lumen on endocytosis dequenched the fluorescence of the cypHer dye and led to a punctate staining of synaptic boutons with intensities depending on the experimental procedure (**Fig. 1d**, **Supplementary Note 2** and **Supplementary Fig. 2**). Neurons were then challenged by 200 or 600 action potentials at 20 Hz to release the synaptic vesicles that were stained during stimulation or at rest without (**Fig. 1d**) and with external calcium (**Supplementary Fig. 3**). Under all experimental conditions, the cypHer signal showed a fast decay as a result of neutralization of vesicular fluorophores on exocytosis followed by slower exponential recovery as a result of compensatory endocytosis and re-acidification. The cypHer signals scale with stimulus strength and the time constants of fluorescence recovery are consistent with previous estimates of endocytic rates[12]. The normalized fluorescence profiles for boutons labeled either spontaneously or during stimulation overlapped perfectly, indicating that both labeled synaptic vesicle pools could be depleted by stimulation to the same degree. Inhibitory synapses labeled with cypHer-coupled antibodies against the vesicle GABA transporter showed identical stimulation-dependent exocytosis of synaptic vesicles labeled spontaneously or by evoked activity, implying that this phenomenon is fundamental and independent of neuron type (**Supplementary Fig. 4**).

The size of the resting pool, generally defined as the pool of synaptic vesicles that cannot be recruited even on strong stimulation, has previously been estimated on the basis of overexpression of synaptopHluorin (spH, the pH-sensitive variant of GFP (pHluorin) coupled

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Received 19 July; accepted 25 October; published online 21 November 2010; doi:10.1038/nn.2695

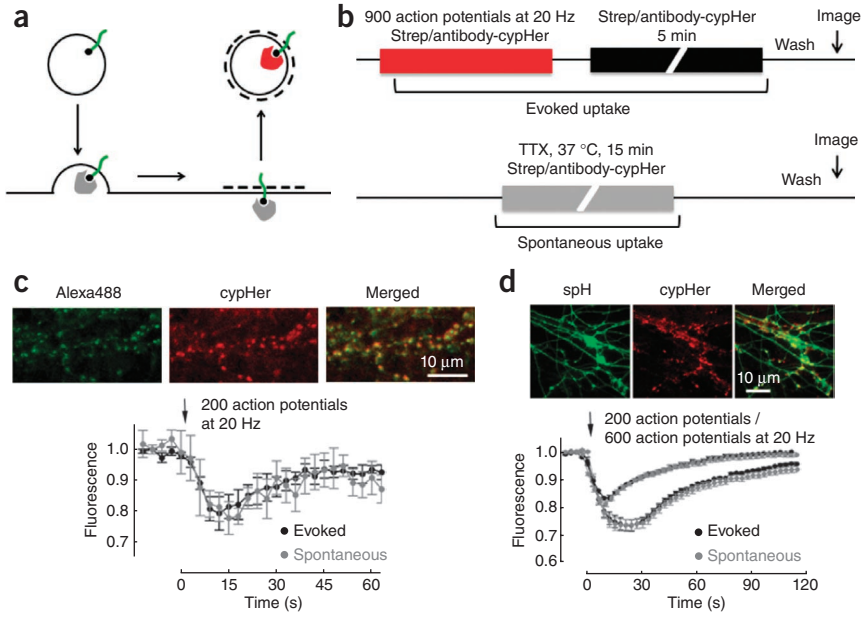

Figure 1 Synaptic vesicles labeled by spontaneous or activity-dependent uptake exhibit identical release kinetics on stimulation. (**a**) On exocytosis, the biotin tag of biosyn or the ectodomain of Syt1 bearing the epitope were exposed to the extracellular space and bind exogenously added cypHer-labeled streptavidin or antibody. CypHer was quenched at the surface and exhibited maximal fluorescence at acidic pH. (**b**) Experimental protocol for labeling selective pools of synaptic vesicles. (**c**) Fluorescence images of hippocampal neurons expressing biosyn labeled with fluorescent monovalent streptavidin showed punctuate bouton staining. CypHer-labeled and Alexa488-labeled streptavidin were added in a ratio of 3:1. Average fluorescence responses of boutons labeled with monovalent cypHer-streptavidin spontaneously or by stimulation with 200 action potentials at 20 Hz were nearly identical. Traces were normalized to the size of the total labeled pool uncovered by a pulse of NH_4Cl at the end of the experiment ($n = 3$ for each condition with >30 boutons per experiment). (**d**) Fluorescence images of spH-transfected hippocampal neurons labeled with αSyt1-cypHer. Average cypHer fluorescence responses to 200 and 600 action potentials at 20 Hz ($n = 4$ with >50 boutons each) revealed that both evoked and spontaneously recycled synaptic vesicles exhibited the same release kinetics. Error bars indicate s.e.m.

to the luminal domain of Syb2)[13] in combination with the alkaline trap method[12]. In this method, the v-ATPase, which re-acidifies the lumen of synaptic vesicle on endocytosis, is blocked by a specific inhibitor, such as folimycin, locking the vesicles in an alkaline state after retrieval. Thus, using depleting stimulation and subsequent superfusion with NH_4Cl, equilibrating the pH across all membranes to 7.4, the fractional size of the recycling pool can be quantified. Previous estimates indicate that there are roughly equal amounts of synaptic vesicles in the recycling and reluctantly releasable resting pools[14]. To determine which fractions of αSyt1-cypHer internalized into synaptic vesicles during spontaneous or evoked activity are recycled back into the recycling or resting synaptic vesicle pools, we applied the alkaline trap method on hippocampal neurons labeled with αSyt1-cypHer either spontaneously or by stimulation. Subsequently, neurons were stimulated with 900 action potentials at 20 Hz in the presence of folimycin and superfused with NH_4Cl to determine the total pool size (**Fig. 2a**). Notably, not only activity-labeled, but also spontaneously loaded, synaptic vesicles could be exocytosed again to the same degree relative to the total signal (**Fig. 2b**), that is, both vesicle types repopulated the recycling and resting pool to the same degree, unlike previous findings[7]. Histogram analysis of fractions of cypHer-labeled synaptic vesicles in the recycling pool revealed almost identical distributions for both spontaneous and activity-dependent labeling (**Fig. 2c**). This implies that synaptic vesicles labeled either during spontaneous or evoked exo-endocytosis recycle equally back into the recycling and resting pools. Pre-silencing of neurons for 30 min with TTX before spontaneous labeling had no effect on the kinetics and distribution of labeled synaptic vesicles to recycling and resting pools, indicating that neuronal activity does not alter release kinetics of spontaneously labeled αSyt1-cypHer on stimulation (**Supplementary Figs. 2** and **5**), contradicting a recent report[15]. These results were confirmed by measurements in dual-color mode of spH-transfected neurons labeled with αSyt1-cypHer (**Fig. 2d**). Because spH tags all of the synaptic vesicles in the presynapse, the corresponding spH traces from the same boutons serve as a reliable internal control.

We next asked whether the recycling pool could be cross-depleted by preceding spontaneous activity, which would further substantiate the claim that both modes of release originate from one pool, that is, the releasable recycling pool. To address this issue, we used

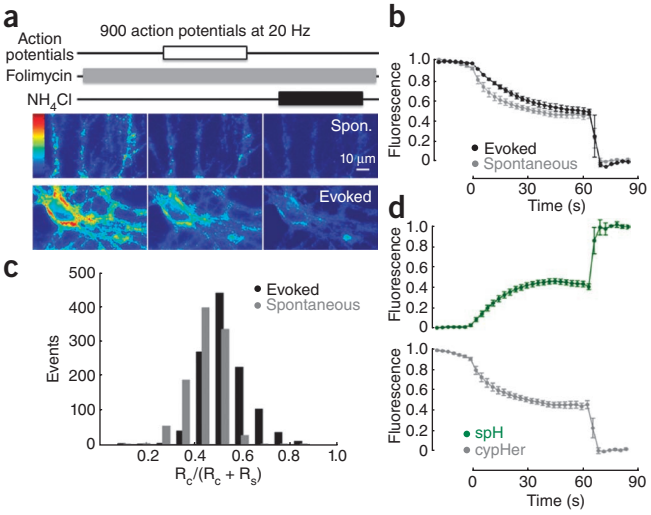

Figure 2 Synaptic vesicles endocytosed spontaneously and on stimulation recycle equally to the recycling (R_c) and resting (R_s) synaptic vesicle pools. (**a**) Fluorescence images of hippocampal boutons labeled with αSyt1-cypHer by activity or spontaneously before (left) and after stimulation (900 action potentials at 20 Hz) in the presence of folimycin (middle), as well as during quenching of nonreleased dye with NH_4Cl (right). (**b**) Corresponding average normalized fluorescence responses ($n = 5$ for each condition with >75 boutons per experiment), yielding sizes of the recycling (R_c) and resting (R_s) pool. (**c**) Histogram of relative recycling pool sizes (as a fraction of the total pool size) for boutons labeled during stimulation (black) or spontaneously (gray). (**d**) Dual-color measurement of spH-transfected neurons colabeled at rest with αSyt1-cypHer following the protocol in **a** ($n = 4$ with >75 boutons each). Error bars indicate s.e.m.

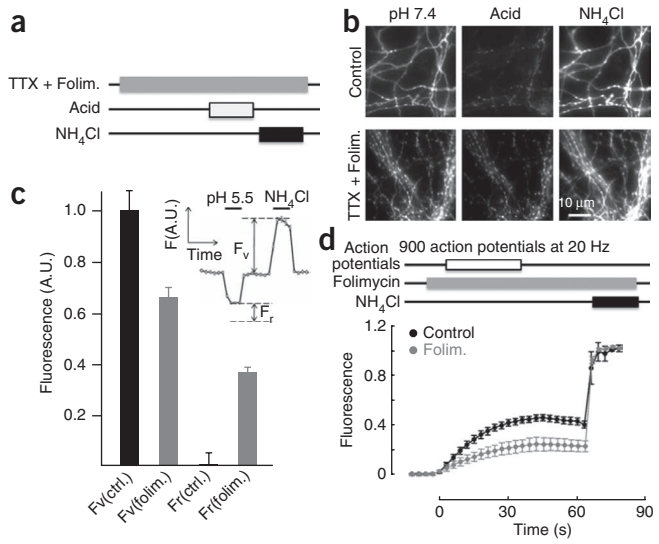

Figure 3 Synaptic vesicles undergoing spontaneous and activity-dependent recycling originate from the recycling pool. (**a**) Schematic of the experimental design. (**b**) Fluorescence images of spH-transfected neurons with or without 15–20 min TTX and folimycin pre-incubation. A brief pulse of acid (pH 5.5) and NH_4Cl was applied to determine the spontaneously released spH fraction (F_r) and the unreleased vesicular spH fraction (F_v). (**c**) Fluorescence amplitudes of F_r and F_v from **b** normalized to the total vesicular fluorescence in control conditions ($n = 6$ for each condition with >75 boutons per experiment). (**d**) Experimental protocol and normalized average spH fluorescence responses to 900 action potentials at 20 Hz and successive NH_4Cl perfusion with or without pre-incubation in TTX and folimycin for 15–20 min ($n = 6$ with >75 boutons). The cross-depletion demonstrates that most spontaneously recycled synaptic vesicles were drawn from the recycling pool. Error bars indicate s.e.m.

spH-transfected neurons and irreversibly dequenched synaptic vesicles undergoing spontaneous exo-endocytosis during 15–20 min by folimycin (**Fig. 3a**). Thus, all of the synaptic vesicles that spontaneously recycled during this time were trapped in the alkaline state, leading to an increase in bouton fluorescence (**Fig. 3a**,**b**). Short superfusion with acidic solution (pH 5.5) allowed us to subtract the nonvesicular spH surface fluorescence from the vesicular one and to quantify the fraction of spontaneously recycled synaptic vesicles (F_r; **Fig. 3b**). The remaining vesicular spH fraction (F_v) not released during the 15–20-min period could be visualized and quantified by a brief NH_4Cl pulse (**Fig. 3b**). Comparison of F_r and F_v between control and TTX/folimycin-treated boutons revealed an increase in F_r and a consequent decrease in F_v to about 65% of control (**Fig. 3c**). We next challenged the neurons with 900 action potentials at 20 Hz in the presence of folimycin to deplete the remaining recycling pool and unmasked the resting pool with NH_4Cl (**Fig. 3d**). We found that the fraction of the recycling pool relative to the remaining total pool decreased from 45% to 25%, indicating that the size of the recycling pool was effectively reduced by preceding spontaneous activity in presence of folimycin (**Supplementary Note 3**). Based on the ratio and the relative decrease of the total vesicular pool to ~65% (**Fig. 3c**), we were able to determine that ~80% (slow mixing between pools undermines this ratio) of spontaneously recycling synaptic vesicles were released from the recycling pool, which is consistent with our claim of one pool of synaptic vesicles supporting both mini and evoked release.

Using three different techniques that combine exogenous labeling of a select pool of synaptic vesicles and/or endogenous labeling of the entire vesicle population, we found that spontaneously recycling synaptic vesicles are depleted by subsequent stimulation, excluding the existence of two different synaptic vesicle pools, recycling and resting, for evoked and miniature activity, respectively. Moreover, by detailed analysis of the repopulation of the resting pool by recycling synaptic vesicles, we found that equal fractions of spontaneously or actively labeled synaptic vesicles entered both the recycling and resting pools. Finally, using spH in a cross-depletion experiment, we observed that more than 80% of the synaptic vesicles responsible for minis were drawn from the recycling pool, and not from the reserve pool. These results imply that the recycling pool of synaptic vesicles in hippocampal synapses is predominantly responsible for spontaneous release and that the reluctantly releasable resting pool instead functions as a reservoir for homeostatic control of presynaptic function. This is corroborated by the recent finding that the resting synaptic vesicle pool can be easily unlocked for evoked release by downregulation of a single kinase[14] (**Supplementary Discussion**).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank K. Hardes for culturing hippocampal neurons, H. Martens (Synaptic Systems) for providing antibodies and E. Neher for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Kl 1334/1-1 to J.K.). Y.H. is supported by a stipend from the Max Planck foundation and R.S. by a stipend from the International Max Planck Research School in Neurosciences Göttingen.

AUTHOR CONTRIBUTIONS

Y.H. and R.S. designed, performed and analyzed the CypHer antibody and spH experiments. M.M. and M.K. designed, performed and analyzed the streptavidin-cypHer experiments. J.K. initialized the project. All of the authors wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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