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A common origin of synaptic vesicles undergoing

evoked and spontaneous fusion

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There is an ongoing controversy on the identity of synaptic vesicles (SVs) undergoing spontaneous versus evoked release. A recent study, introducing a new genetic probe, suggested spontaneous release to be driven by a resting pool of SVs refractory to stimulation. Here we demonstrate cross-depletion of spontaneously or actively recycling SV pools upon stimulation in rat hippocampal neurons and identify the recycling pool as 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 transmission¹ These action potential (AP)-independent minis were considered to arise from low-probability fusion of single SVs (about one per 90 s)², that are docked and primed for release. Newer data on the Ca^{2+} -activation of vesicle fusion indeed indicate that spontaneous transmitter release close to resting $[Ca^{2+}]_i$ is a mere 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 most recently proposed that the resting pool of SVs, usually refractory to stimulation, is mobilized during spontaneous activity and is hence responsible for minis ⁷. The authors introduced a novel genetic probe termed biosyn (biotinylated Synaptobrevin 2), to tag SVs in hippocampal neurons based on biotinylation of the SV protein synaptobrevin2 (syb2) which then could be labeled by fluorescent streptavidins when exposed to the surface, i.e. during SV recycling. The major drawback of this technique, however, is that only release can be monitored but not the fate of labeled SVs upon a further round of stimulation, i.e. not to which pool of SVs they recycle. To resolve this 'one-shot experiment' problem, we refined the biosyn approach and coupled a new pH-sensitive cyanine dye, cypHer 5E⁹ to unlabeled monovalent streptavidin ¹⁰ avoiding possible impairment because of cross-linking introduced by the tetravalent wildtype probe (**Supplementary Fig. 1, Supplementary Note 1, Supplementary Material and Methods**). The fluorescence of the cypHer dye is maximal at intravesicular pH of 5.5 and is almost quenched at slightly basic extracellular pH of 7.4 after SV exocytosis (**Fig. 1a**). Using cypHer-streptavidin, we now could label SVs in live hippocampal boutons expressing biosyn either by stimulation (900 APs at 20 Hz to mobilize the entire recycling pool) or by

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spontaneous activity (15 min at 37°C in presence of the sodium-channel inhibitor tetrodotoxin (TTX)) (Fig. 1b). Neurons were then stimulated with 200 APs at 20 Hz to test the release-competence of SVs which 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 upon stimulation with near-identical relative amplitudes followed by a slow increase due to compensatory endocytosis. Therefore, spontaneously and stimulation-labeled SV pools can be depleted by stimulation to the very same degree suggesting one common SV pool.

We confirmed these results using live hippocampal boutons labeled with an exogenous cypHercoupled antibody ¹¹ against the luminal domain of the SV protein synaptotagmin1 (Syt1: α Syt1-cypHer) following both protocols described above (Fig.1b). Reacidification of the intravesicular lumen upon endocytosis dequenches the fluorescence of the cypHer dye and leads to a punctate staining of synaptic boutons with intensities depending on the experimental paradigm (Fig. 1d, Supplementary Note 2, Supplementary Fig. 2). Neurons were then challenged by 200 or 600 APs at 20 Hz to release the SVs stained during stimulation or at rest without (Fig. 1d, bottom) and with external calcium (Supplementary Fig. 3). Under all experimental conditions the cypHer signal showed a fast decay due to neutralization of vesicular fluorophores upon exocytosis followed by slower exponential recovery due to compensatory endocytosis and reacidification. The cypHer signals scale with stimulus strength and the time constants of fluorescence recovery are consistent with previous estimates of endocytic rates ¹². The perfectly overlapping normalized fluorescence profiles for boutons labeled either spontaneously or during stimulation show that both labeled SV pools can be depleted by stimulation to the very same degree. Inhibitory synapses labeled with cypHer-coupled antibodies against the vesicle GABA transporter (VGAT; αVGAT-cypHer) showed identical stimulation-dependent exocytosis of SVs labeled spontaneously or by evoked activity implying that this phenomenon is rather fundamental and independent of neuron type (Supplementary Fig. 4).

The size of the resting pool, generally defined as the pool of SVs that cannot be recruited even upon strong stimulation has previously been estimated based on overexpression of synaptopHluorin (spH, the pH-sensitive variant of GFP (pHluorin) coupled to the luminal domain of syb2)¹³ in combination with the alkaline trap method ¹². In this method, the v-ATPase, that reacidifies the lumen of SV upon endocytosis, is blocked by a specific inhibitor, such as folimycin, locking the vesicles in an alkaline

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state after retrieval. Thus, by depleting stimulation and subsequent superfusion with NH₄Cl, equilibrating the pH across all membranes to 7.4, the fractional size of the recycling pool can be quantified. Previous estimates indicate about equal amounts of SVs in the recycling and reluctantly releasable resting pools ¹⁴. To determine what fractions of α Syt1-cypHer antibodies internalized into SVs during spontaneous or evoked activity are recycled back into the recycling or resting SV pools we applied the alkaline trap method on hippocampal neurons labeled with aSyt1-cypHer either spontaneously or by stimulation as above. Subsequently, neurons were stimulated with 900 APs at 20 Hz in the presence of folimycin and superfused with NH₄Cl to determine the total pool size (Fig. 2a). Surprisingly, not only activity-labeled but also spontaneously loaded SVs could be exocytosed again to the same degree relative to the total signal (Fig. 2b), i.e. repopulated the recycling and resting pool to the same degree, unlike reported recently⁷. Histogram analysis of fractions of cypHer-labeled SVs in the recycling pool revealed almost identical distributions for both spontaneous and activity-dependent labeling (Fig. 2c). This implies that SVs 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 prior to spontaneous labeling had no effect on kinetics and distribution of labeled SVs to recycling and resting pools indicating that neuronal activity does not alter release kinetics of spontaneously labeled α Syt1-cypHer upon stimulation (**Supplementary Fig. 2 and 5**), contradicting a recent report¹⁵. These results could be confirmed by measurements in dual-color mode on spH-transfected neurons labeled with α Syt1-cypHer (Fig. 2d). Since spH tags all SVs in the presynapse, the corresponding spH traces from the same boutons serve as a reliable internal control for the above experiment.

Next we probed, 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, i.e. the releasable recycling pool. To address this issue we used spH-transfected neurons and first irreversibly dequenched SVs undergoing spontaneous exo-endocyosis during 15-20 min by folimycin (**Fig. 3a**). This way all SVs 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) allows to subtract the non-vesicular spH surface fluorescence from the vesicular one and thus to quantify the fraction of spontaneously recycled SVs (F_r) (**Fig. 3a**). The remaining vesicular spH fraction (F_v) not released during the 15–20 min period could be visualized and quantified by a brief NH₄Cl pulse (**Fig. 3b**). Comparison of F_r and F_v between control and TTX/folimycin-treated boutons revealed

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an increase in F_r and a consequent decrease in F_v to about 65 % of control (Fig. 3c). Next, we challenged the neurons with 900 APs at 20 Hz in presence of folimycin to deplete the remaining recycling pool, and finally unmasked the resting pool with NH₄Cl (Fig. 3d). We found that the fraction of the recycling pool relative to the remaining total pool decreased from 45 % to 25 % showing that the size of the recycling pool is effectively reduced by preceding spontaneous activity in presence of folimycin (cf. Supplementary Note 3 for exclusion of a passive proton leak). Based on the ratio and the relative decrease of the total vesicular pool to ~ 65 % (Fig. 3c) we can determine that ~80 % (slow mixing between pools undermines this ratio) of spontaneously recycling SVs are released from the recycling pool, which is in perfect agreement with our claim of one pool of SVs supporting both mini and evoked release.

In this study we have used three different techniques which combine exogenous labeling of a select pool of SVs and/or endogenous labeling of the entire vesicle population to clearly demonstrate the depletion of spontaneously recycling SVs by subsequent stimulation excluding the existence of two different SV pools, recycling and resting, for evoked and miniature activity, respectively. This is corroborated by the recent finding that the resting SV pool can be easily unlocked for evoked release by downregulation of a single kinase ¹⁴ (**Supplementary Discussion**).

Supplementary Information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.H. and R.S. designed, performed and analysed the CypHer-antibody and SpH experiments. M.M and M.K. designed, performed and analysed the streptavidin-cypHer experiments. J.K. initialized the project. J.K. and R.S. together with other authors wrote the paper.

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FIGURE LEGENDS

Figure 1 SVs labeled by spontaneous or activity-dependent uptake exhibit identical release kinetics upon stimulation. (a) Upon exocytosis, the biotin tag of biosyn or the ectodomain of Syt1 bearing the epitope are exposed to the extracellular space and bind exogenously added cypHer-labeled streptavidin or antibody. CypHer is quenched at the surface and exhibits maximal fluorescence at acidic pH. (b) Experimental protocol for labeling selective pools of SVs. (c) Fluorescence images of hippocampal neurons expressing biosyn labeled with fluorescent monovalent streptavidin show

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 to 200 APs at 20 Hz are nearly identical. Traces were normalized to the size of the total labeled pool uncovered by a pulse of NH_4CI 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 α Syt1-cypHer. Average cypHer fluorescence responses to 200 and 600 APs at 20 Hz (n = 4 with > 50 boutons) reveal that both evoked and spontaneously recycled SVs exhibit the same release kinetics. All errors in s.e.m

Figure 2 SVs endocytosed spontaneously and upon stimulation recycle equally to the recycling (R_c) and resting (R_s) SV pools. (**a**) Fluorescence images of hippocampal boutons labeled with α Syt1-cypHer by activity or spontaneously before (left) and after stimulation (900 APs at 20 Hz) in the presence of folimycin (middle) as well as during quenching of non-released dye with NH₄Cl (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 fraction of the total pool size) for boutons labeled during stimulation (dark blue) or spontaneously (light blue). (**d**) Dual-color measurement of SpH-transfected neurons co-labeled at rest with α Syt1-cypHer following the protocol in (a) (n=4 with > 75 boutons). All errors in s.e.m.

Figure 3 SVs undergoing spontaneous and activity-dependent recycling originate from the recycling pool. (**a**) Schematics of the experimental designs. (**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 NH₄Cl was applied to determine the spontaneously released SpH fraction (F_r) and the unreleased vesicular SpH (F_v) fraction. (**c**) Fluorescence amplitudes of F_r and F_v from experiments in (a,b) normalized to the total vesicular fluorescence in control conditions (n = 6 for each condition with > 75 boutons per experiment). (**d**) Normalized average SpH fluorescence responses to 900 APs at 20 Hz and successive NH₄Cl perfusion with or without pre-incubation (n = 6 with > 75 boutons). The cross-depletion demonstrates that most spontaneously recycled SVs were drawn from the recycling pool. All errors in s.e.m





