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Title: Ageing synaptic vesicles are inactivated by contamination with SNAP25

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Abstract: Old organelles can become a hazard to cellular function, by accumulating molecular damage. Mechanisms that identify aged organelles, and prevent them from participating in cellular reactions, are therefore necessary. We describe here one such mechanism, which acts as a timer that inactivates aged synaptic vesicles. Using cultured hippocampal neurons, we found that newly synthesized vesicle proteins are incorporated in the active (recycling) pool, and are preferentially employed in neurotransmitter release. They remain in use for up to ~24 hours, during which they recycle ~200 times, on average. Over this period the vesicles of the active pool become contaminated with the plasma membrane protein SNAP25, which inhibits the vesicle-associated chaperone CSPα. This renders these used vesicles less competent to release than newly synthesized ones that lack SNAP25. The old and contaminated vesicles are eventually targeted for degradation, possibly through the direct involvement of SNAP25. This timer mechanism can be circumvented by over-expressing CSPα, which, however, leads to less efficient recycling, and to neurite degeneration.

This submission includes:
- Main Text (5174 words), including Main Figure Legends (1676 words)
- 7 Main Figures
- 8 Supplementary Figures
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INTRODUCTION

Dividing cells are continuously replaced in their entirety, and often remain fully functional even at the end of an organism's lifespan. In contrast, non-dividing cells, including neurons, accumulate damage to their organelles, which can manifest itself as disease[1,2]. To prevent detrimental effects, these cells need to avoid the use of aged and damaged organelles, which could disrupt cellular function in unpredictable ways. Such mechanisms have been described, for example, in budding yeast, where old and damaged mitochondria and vacuoles are retained in the ageing mother cell, to prevent their usage in the daughter cells[3].

Most mechanisms described so far target damaged components of the organelles, and not their actual age. The damage is dealt with by degrading the organelles, or by sorting out the damaged components. This scenario can function well for cellular processes in which many organelles function in parallel, and where damage to one organelle is not endangering the outcome of the entire process. It would, however, not be efficient for signaling processes that rely on only a handful of organelles at any one time, such as synaptic vesicle exocytosis[4–7]. Such processes could be severely disrupted by single damaged vesicles. If one or more of the synaptic vesicles docked at an active zone fail to release, the synapse remains silent, and fails to transmit the signal. Even more importantly, if released vesicles fail to recycle correctly, they might fail to liberate the active zone, which would result in a persistent inhibition of the synapse[8]. This suggests that neurons should have mechanisms in place that recognize old, damage-prone vesicles, to inhibit the use of such vesicles in synaptic release, even before they accumulate substantial levels of damage.

Some evidence in support of this hypothesis exists in non-neuronal secretory cells, including chromaffin cells[9] and pancreatic β-cells[10]. Here, newly synthesized dense-core vesicles, which are not recycled after use, can be released during mild cellular activity, but aged ones only exocytose after heavy artificial stimulation. The situation affecting synaptic vesicles, which recycle repeatedly within the synaptic boutons, is less clear. Here, the vesicles can be broadly separated into 1) an active recycling pool, which includes the readily releasable vesicles that are docked at the release sites, and 2) an inactive reserve pool that participates little in release under most stimulation conditions, and can typically only be released by heavy artificial stimulation[11]. It would be tempting to hypothesize that the situation mirrors that from non-neuronal secretory cells, with active vesicles being newly synthesized ones, and inactive vesicles being aged ones. However, there is so far no evidence either for or against this hypothesis.

A further issue complicates this interpretation: the problem of the vesicle identity[8,12,13], for which two opposing models have been presented. In a first model, the vesicle maintains its protein composition after exocytosis, as a single patch of molecules on the plasma membrane, which is then retrieved as a whole by endocytosis. In the second model, the vesicle loses its molecular cohesion upon fusion, and its proteins diffuse in the plasma membrane and intermix with other vesicle proteins, before endocytosis. In the first scenario, the neuron could readily target old vesicles for removal. This is less obvious in the second scenario, since vesicles lose their identity by intermixing molecules, which makes it more difficult to pinpoint the old vesicle components.

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Although it has been difficult to differentiate between these two scenarios, an unified view is starting to emerge. This view suggests that several synaptic vesicle proteins remain together during recycling, as meta-stable molecular assemblies, although not as whole individual vesicles[13]. In simple terms, this view implies that the vesicle splits into a number of meta-stable protein assemblies after exocytosis. These individual assemblies are stabilized by strong interactions between abundant vesicle proteins such as synaptophysin and synaptobrevin/VAMP2[14–17], are strong enough to persist even after detergent solubilization[18], and may be further stabilized by an interaction with the endocytosis machinery[19]. The assemblies have been observed by all laboratories that have studied this issue using super-resolution imaging (see for example[20–24]), and are fully compatible with modern interpretations on the meta-stable nature of membrane protein assemblies (for example[25]). The vesicle protein assemblies are then regrouped during endocytosis and vesicle reformation, resulting in new synaptic vesicles.

Could such a scenario enable a neuron to nevertheless distinguish between old and young vesicles? This seems unlikely at the level of the single vesicles, but entirely possible at the level of the vesicle pools. The active and inactive synaptic vesicles maintain their pool identities over long time periods (see review[11]), and it has been thoroughly demonstrated that mild (physiological) stimulation results in no molecular mixing among vesicles from different pools[24]. Therefore, as long as the recycling vesicles only intermix molecules with other recycling vesicles, they remain separated from the reserve ones, and can be specifically targeted and/or timed by the neurons. At the same time, the meta-stable nature of the vesicle protein assemblies implies that such assemblies could become contaminated over time with non-vesicle molecules, which could tag them as old, used molecular patches.

The hypothesis that neurons have mechanisms to recognize old and damage-prone vesicles is thus plausible, irrespective of whether the vesicle fully maintains its identity during recycling, or splits into meta-stable assemblies that intermix within one single pool of vesicles (within the recycling pool). We set out to test this hypothesis here, and found that the ageing recycling vesicle proteins were indeed identified by the neurons. Moreover, after their proteins went through ~200 rounds of recycling (on average) they were inhibited from participation in neurotransmitter release, through a mechanism involving the SNARE SNAP25 and the chaperone CSPα.

RESULTS

Synaptic vesicles become inactive as they age

We first verified the behavior of the proteins from the recently exocytosed vesicles. As discussed in the Introduction, we expected that assemblies of vesicle molecules, containing different types of vesicle proteins, would appear on the plasma membrane after exocytosis, and that only a minority of the proteins would diffuse away and be lost in the membrane, far from the assemblies. To test this, we tagged the synaptic vesicle proteins synaptophysin and synaptotagmin 1 from newly exocytosed vesicles, using fluorescently conjugated antibodies, and quantified their co-clustering by two-color super-resolution STED microscopy[20] (Supplementary Fig. 1a). We observed a very limited loss of proteins from the synaptic vesicle protein assemblies (co-clusters), around 3% (Supplementary Fig. 1b,c). As

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mentioned in the Introduction, this is in line with observations from several different groups, especially for low stimulation levels that are comparable to the intrinsic network activity of the cultures[20–24,26].

To follow synaptic vesicle proteins through their life cycle in the synapse, we used fluorophore-conjugated antibodies directed against the lumenal domains of the vesicle proteins synaptotagmin 1[27,28] and VGAT[29], in living, active rat primary hippocampal cultures (Fig 1a). These are the only such antibodies currently available. The synaptophysin antibodies used in Supplementary Fig. 1 do not recognize the unfixed protein, and cannot be used in this assay. The antibodies were taken up during the intrinsic network activity of the cultures, and remained bound to their target proteins for up to ten days (Fig. 1b; Supplementary Fig. 2b,d). Incubating neurons with the antibodies for one hour tags the entire active recycling pool of vesicles, which accounts for approximately 50% of all vesicles at the synapse (Supplementary Fig. 2c). The vesicles were slowly lost from the synapses (Fig. 1c), and were degraded in lysosomes in the cell body (Supplementary Fig. 2e,f). The turnover of both synaptotagmin 1 (Fig. 1b,c) and VGAT (Fig. 1c) was well within the range previously measured for synaptic vesicle proteins by radioisotopic labelling or mass spectrometry[30,31].

To test whether the tagged proteins of different ages were still involved in synaptic activity, we employed antibodies conjugated to the pH-sensitive dye CypHer5E[21,29]. We used a stimulation protocol designed to trigger the release of the entire population of releasable vesicles, 600 action potentials at 20 Hz[32], and we measured the fraction of the CypHer5E-labeled proteins that were releasable (Fig. 1d). This was monitored through imaging the stimulation-induced reduction in CypHer5E fluorescence, which is quenched at neutral pH, and therefore becomes invisible upon exocytosis. We found that the fraction of the labelled proteins that could be induced to release decreased with age, until almost no response could be elicited any more, at 7-10 days after tagging (Fig. 1d).

We also investigated this phenomenon during intrinsic network activity, focusing on synaptotagmin 1 (since the low proportion of VGAT-positive neurons, ~5-10%, is likely not representative for the general activity of our cultures). The decrease of the releasable fraction occurred even faster, and the majority of antibody-tagged proteins stopped participating in release within one day (time constant of ~0.4 days; Fig. 1e; Supplementary Fig. 3c). After this first day, many of the vesicle proteins were still present in the synapse (Fig. 1b,c; Supplementary Fig. 3b,c), and could be triggered to release by strong stimulation (Fig. 1d), but did not release under normal network activity (Fig. 1e, Supplementary Fig. 3b,c). We termed this the “inactive state” of synaptic vesicles, or vesicle molecules. It could also be called a “reserve” or “reluctant” state: the vesicles are present in the synapse, release upon supra-physiological stimulation, but not during normal activity. The inactivity (or reluctance) became absolute after about 7-10 days (Fig. 1d).

A sensor for the age of vesicle proteins confirms that young molecules are preferentially employed in exocytosis

To complement these antibody-based approaches, we tested the behavior of the synaptic vesicle protein VAMP2 after tagging with a novel construct that enables the separate identification of newly synthesized or older proteins. We expressed VAMP2 coupled to a SNAP tag[33,34], and separated from the original sequence by a TEV protease cleavage site: VAMP2-TEV-SNAPtag (Supplementary Fig. 4a). This construct should be minimally

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disruptive to physiological synaptic vesicle function, since VAMP2 is by far the most abundant synaptic vesicle protein[35,36], and therefore every vesicle should still have ample levels of wild-type VAMP2, independent of the levels of VAMP2-TEV-SNAPtag expression. Moreover, our construct is designed on the basis of synaptopHluorin (VAMP2-pHluorin), which is known to target and function well in neurons[37,38]. We expressed this construct in hippocampal cultures, and applied a first pulse of a membrane-permeable fluorophore (TMR-Star) to saturate all available SNAP tags. The coupling reaction is self-catalyzed by the SNAP tag[39]. After 1-2 days in culture, we applied a second pulse of a spectrally separable fluorophore (647-SiR), to label newly produced vesicles. The neurons thus contain two populations of VAMP2-TEV-SNAPtag, one young and one 1-2 days older, which have different colors (Supplementary Fig. 4b). We then added the TEV protease to living, active cultures, to cleave the labelled SNAP tags off the releasing vesicles. Inactive vesicles are not affected by the TEV protease, since it cannot penetrate the cell membrane. Confirming the observations from Fig. 1, we observed that the tag was cleaved preferentially from young VAMP2 proteins (Supplementary Fig. 4b,c).

Releasable synaptic vesicles are metabolically younger than inactive vesicles

Having verified with two independent techniques that newly synthesized protein copies are preferentially used in exocytosis for three vesicle proteins (synaptotagmin 1, VGAT, VAMP2), we proceeded to test whether the entire protein makeup of actively recycling vesicles is metabolically younger than that of non-recycling, inactive vesicles. We tagged newly synthesized proteins with amino acids that can be detected either by fluorescence microscopy or by imaging mass spectrometry (nanoSIMS). We fed the cultures the unnatural amino acid azidohomoalanine (AHA), or leucine containing the stable nitrogen isotope ^{15}N. We then correlated these amino acids, which serve as markers for the protein age, with antibody tags for the releasable or inactive synaptic vesicle (Figure 2a). AHA incorporates into all newly produced proteins in the position of methionine, and can be detected in fluorescence microscopy after fluorophore conjugation to AHA (FUNCAT[40]), through CLICK chemistry after fixation[41]. ^{15}N-leucine can be detected through the mass spectrometry imaging technique nanoSIMS[42,43], which has a higher resolution than conventional fluorescence microscopy (~50-100 nm in cultured neurons[44]). We correlated the ^{15}N signal to the positions of synapses and vesicles, by first imaging the preparations in fluorescence microscopy, and then in nanoSIMS (COIN[44]).

In both approaches, we could detect a significantly higher co-localization of the actively recycling synaptic vesicles with newly synthesized proteins (Fig. 2b,c). Moreover, the higher sensitivity of nanoSIMS, which, unlike FUNCAT, detects simultaneously both the ^{14}N from old proteins and the ^{15}N from new proteins, could demonstrate that the active vesicles were significantly younger than the rest of the axon. The opposite was true for the inactive vesicles (Fig. 2c).

Inactivated vesicles cannot replace young vesicles in the releasable population

This hypothesis (the low intermixing of old and young vesicle proteins) was further confirmed by the observation that inactivated vesicles cannot be recruited to replace young vesicles in the active, releasable population during intrinsic network activity (Fig. 3). To test this, we applied unconjugated lumenal synaptotagmin 1 antibodies to saturate all of the epitopes of the releasable population (Fig. 3a), and then followed this up with pulses of fluorophore-conjugated synaptotagmin 1 antibody, to reveal new synaptic vesicles entering the active,

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releasable population (Fig. 3b,c). These vesicles could come from two sources: newly synthesized vesicles from the cell body, or the inactivated vesicles, whose epitopes are not affected by the initial incubation with unconjugated antibodies, and which account for ~50% of all of the vesicles in the synapse (Supplementary Fig. 2c). Cutting off the production of new synaptic vesicles by blocking protein biosynthesis with anisomycin, or by disrupting the microtubule transport network with colchicine, completely removed the entry of new epitopes into the releasable population (Fig. 3b,Cc).

This indicates that, under conditions of intrinsic network activity, the synapse is dependent on young vesicles to replace its releasable population. The drugs did not significantly affect the proportion of releasable vesicles in the synapse, the intrinsic network activity, or the total amount of vesicles per bouton (Fig. 3d-f), suggesting that the neurons were still healthy at the time of the experiments.

Increased synaptic activity accelerates ageing and inactivation

We next investigated whether temporal age is the defining factor for inactivating vesicles, or whether the usage of the vesicles (or vesicle proteins) is responsible, and, if the latter is true, how often proteins from the vesicles could be used before inactivation.

If the age of the vesicle proteins is the defining parameter in the inactivation, then increasing the frequency of vesicle release and recycling should have no influence on the rate of inactivation. If, however, the vesicle protein usage controls the inactivation, then chronically increased synaptic activity should lead to a faster inactivation. We tested therefore the speed of inactivation (as in Supplementary Fig. 3), in the presence of the $GABA_A$ receptor antagonist bicuculline, or with a Ca^{2+} concentration raised to 8 mM, for 12 hours. Both treatments lead to a chronic increase in synaptic activity (~2-fold, see Supplemental Experimental Procedures), and in both cases synaptic vesicles shifted from the releasable state to the inactive state more rapidly (Fig. 4). This implies the existence of a mechanism that acts as a timer for synaptic vesicle release, and inactivates the “used” vesicles after a certain average number of release rounds.

This number can be estimated by measuring three essential parameters: the frequency of synaptic activity in culture during undisturbed network activity, the percentage of the vesicle proteins from the active pool that recycle during each synaptic activity burst, and the amount of time spent by the vesicle proteins in the releasable population. We already obtained the last parameter from Supplementary Fig. 3c. The neuronal activity rate was measured by monitoring neuronal activity bursts, using the calcium indicator construct GCaMP6[45] (Fig. 5a-c). The frequency of the bursts of activity was ~0.09 Hz (Fig. 5c), and each burst consisted of multiple action potentials at high frequency (about 60-80, Fig. 5d). To estimate the percentage of the vesicle proteins released per activity burst, we performed simultaneous imaging of GCaMP6 and the synaptophysin-based pHluorin sypHy[46,47], as an indicator of synaptic vesicle release (Fig. 5e-g). Synaptic vesicle release robustly coincided with Ca^{2+} bursts (Fig. 5f,g). Each burst triggered the release of ~2-3% of all sypHy molecules, on average (Fig. 5h,i). From the activity burst frequency, the protein fraction released per activity burst, and the time constant of inactivation (Supplementary Fig. 3), one can calculate the average number of release events that a set of vesicle molecules undergoes under intrinsic network activity conditions. This averages to ~210 rounds of release (see Online Methods for the calculation).

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Inactivation is triggered by contamination with SNAP25

A timer mechanism that can estimate the number of release events could be based on changes in the composition of the vesicles. Small amounts of proteins could be eventually lost or gained from the vesicle protein assemblies during recycling (see Supplementary Fig. 1 and the introductory paragraphs), thereby resulting in changes in the composition of the recycling vesicles. We immunostained vesicles of different ages and functional states for several candidate proteins, and imaged them by 2-colour STED microscopy, in ultrathin sections. We chose the candidate proteins based on their abundance[35,36], based on their importance in synaptic vesicle release[13,48], and based on their presence in compartments involved in synaptic vesicle release and recycling[13,35]. The following candidate proteins were tested: SNAP25 and syntaxin 1 for the cell membrane, VGlut 1/2, vATPase, VAMP2 and synaptotagmin 1 for the synaptic vesicles themselves, syntaxin 16 and VAMP4 for endosomal compartments, and synapsin as an abundant soluble vesicle-associated protein. We detected only one significant change: SNAP25 gets enriched in ageing, inactive synaptic vesicles (Fig. 6a), which have approximately 2-fold more SNAP25 than young ones. This presumably takes place via SNAP25 being picked up during vesicle retrieval from the plasma membrane, where SNAP25 is present at a 6-7 fold higher density (per μm^2 of membrane) than in synaptic vesicles[35,36].

To test whether the contamination with SNAP25 could have functional consequences, we engineered a construct to specifically target SNAP25 to synaptic vesicles: sypHy-SNAP25, a simple fusion of the synaptophysin-based pHluorin sypHy and SNAP25. Synaptophysin targets to synaptic vesicles more reliably than most other proteins[36,49], and can therefore efficiently incorporate SNAP25 into the vesicles. The pHluorin moiety enabled us to directly observe the response of these vesicles to stimulation. Exocytosis was severely suppressed by the addition of SNAP25 on the vesicles (Fig. 6b). This suggests that the SNAP25 contamination on aged synaptic vesicles is sufficient to inactivate them.

SNAP25 inactivates synaptic vesicles by blocking CSPα

To answer the question of how the presence of SNAP25 on aged synaptic vesicles might inactivate them, we used the known copy numbers of synaptic vesicle proteins[35,36] to predict the most likely SNAP25 target protein. The best-known SNAP25 interactor on the vesicle is VAMP2. SNAP25 on the cell membrane interacts in *trans*-complexes with VAMP2 on the vesicle during exocytosis, so it could, in principle, be envisioned that SNAP25 on the vesicles might block all copies of VAMP2 on the vesicle in *cis*-SNARE complexes. However, VAMP2 is present in ~70 copies per vesicle[35,36], while we estimate a maximum of ~5 SNAP25 copies on the aged vesicles, assuming that vesicles start out with 1-2 copies of SNAP25[36], an amount which then doubles during ageing (Fig. 6a). Five SNAP25 copies are unlikely to result in sufficient interference with 70 copies of VAMP2.

SNAP25 is also known to interact with CSPα, a chaperone needed to prepare the fusogenic *trans*-complex of SNAP25 and VAMP2[48,50]. There are only 2-3 copies of CSPα on each vesicle[35,36]. This value is far closer to our estimated number of SNAP25 copies in aged vesicles, and it is therefore conceivable that SNAP25 might sequester all of them in non-fusogenic *cis*-complexes on the vesicle surface. Normally, a *trans*-complex forms between vesicular CSPα, SNAP25 from the plasma membrane, and two soluble molecules, the ubiquitous chaperone Hsc70 and SGTα[51]. This *trans*-complex is involved in priming SNAP25

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for fusion. The formation of this complex in *cis*, on the vesicle surface, would create a quantitative bottleneck for the fusion of the aged vesicles in the form of sequestration of CSPα. According to this hypothesis, overexpressing CSPα would remove this bottleneck, and would thus remove the timing mechanism that inactivates ageing synaptic vesicles.

Overexpression of CSPα indeed resulted in the release of almost all vesicles available at the synapse (Fig. 7a,b). Old and young vesicles are functionally indistinct in this situation, presumably because the accumulation of SNAP25 on the ageing vesicles is insufficient to block the overexpressed CSPα effectively during the time of the experiment. We did not observe any change in activity levels when overexpressing a mutated form of CSPα that does not target to vesicles correctly[52] (Fig. 7b). The opposite effect, a decrease in synaptic release, was observed when overexpressing SNAP25 (Fig. 7a,b). This effect, which is in agreement with previous findings on SNAP25 overexpression[53], is specific to SNAP25, since expressing the other plasma membrane SNARE, syntaxin 1, results in a strong increase of synaptic release, as expected when raising the copy numbers of a fusion protein (Fig. 7b). Finally, CSPα overexpression also counteracted the effects of the SNAP25 overexpression, as predicted by our hypothesis (Fig. 7a,b).

The elimination of vesicle ageing by CSPα overexpression, however, was deleterious to the cells, suggesting that the inactivation of old vesicles is physiologically relevant. First, the cells overexpressing CSP_{WT} had a significantly higher proportion of damaged neurites than those expressing CSP_{mut} that does not target to vesicles (Supplementary Fig. 5a,b). Second, the endocytosis of aged vesicles appeared to be poorer than that of young vesicles (Supplementary Fig. 5c,d). These effects mirror those found in mice lacking CSPα[54], which suffer from neurodegeneration, and have endocytosis defects that were difficult to reconcile with a role of CSPα in SNAP25 priming. Our results explain this latter finding, since old vesicles would continue to recycle in the absence of CSPα, leading to defects in vesicle recycling. Thus, overexpression and deletion of CSPα lead to some similar phenotypes. The reason for this paradoxical effect is that both overexpression and deletion of CSPα remove the timer mechanism that inactivates ageing vesicles.

The inactivated synaptic vesicles are eventually degraded. We hypothesized that this may occur through the direct participation of SNAP25. Synaptic vesicle degradation is widely assumed to entail fusion to the endo-lysosomal system[13,55,56]. This fusion event requires a combination of R, Q_a, Q_b, and Q_c SNAREs on the surface of the endosome and of the synaptic vesicle. While vesicular R-SNAREs (VAMP2) and endosomal Q_a SNAREs are present in abundance in the synapse, Q_b- and Q_c-SNAREs are relatively scarce[35]. SNAP25 is a Q_{bc} SNARE, and might thus increase the probability of fusing the vesicles to the endo-lysosomal system, as has been previously suggested in PC12 cells[57,58]. We therefore tested whether the overexpression of SNAP25 or sypHy-SNAP25 increases the co-localization of the vesicles with the recycling endosome marker Rab 7, which is thought to be the first step in vesicle degradation. This was observed in both cases (Supplementary Fig. 6).

Predicting synaptic physiology from a quantitative model of the synaptic vesicle protein life cycle

The quantitative data we gathered here allowed us to model the vesicle protein life cycle mathematically (Supplementary Fig. 7). As assumptions for this model, we used the conclusions presented above, *i.e.* that newly synthesized synaptic vesicle proteins start out

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in the releasable population. The synaptic vesicle protein assemblies retrieved upon recycling can then become contaminated with SNAP25, which in turn inactivates the vesicles, and finally forces them to enter the degradation pathway (Supplementary Fig. 7a). The model we constructed from this is based on a series of exponential equations (see Online Methods), and recapitulates the data we presented on synaptic vesicle degradation (Supplementary Fig. 7b). The model thus enabled us to predict the probability distributions of the time it takes to inactivate synaptic vesicle proteins (Supplementary Fig. 7c), the time the inactivated vesicles remain in the inactive population within the synapse before degradation (Supplementary Fig. 7d), the total synaptic vesicle protein lifetime (Supplementary Fig. 7e), and the usage of synaptic vesicle proteins during their lifetime (Supplementary Fig. 7f). The average number of release rounds per synaptic vesicle protein lifetime predicted from the model is ~199, which fits very well with the usage calculated directly from the experimental data above (~210).

DISCUSSION

Organelle ageing has long been recognized as an important factor in cellular disease and death. However, little is known about how aged organelles are identified before they can noticeably impair cellular pathways, especially for post-mitotic cells, which are most strongly affected by malfunctioning organelles. We address here this problem for a well understood organelle, the synaptic vesicle. There is substantial information on how the synaptic vesicle is degraded[13,59–61], but it has never been clear why a fraction of the vesicles are inactivated and are reluctant to participate in neurotransmission[4,11,13,62,63]. We suggest here that this mechanism is the gradual contamination of synaptic vesicles from the recycling (active) pool with SNAP25, during multiple rounds of release and recycling.

This mechanism fits most easily with the concept that the identity of the vesicle is maintained for a relatively long time, throughout multiple rounds of recycling, as discussed in the introduction. However, it also fits with the view of intermixing of vesicle components in the plasma membrane, after exocytosis. In this view, meta-stable vesicle protein assemblies, albeit not full vesicles, can become contaminated with SNAP25 upon recycling. The contaminated assemblies are then incorporated into full vesicles during endocytosis, and the resulting vesicles are thereby tagged as old vesicles, become inactive, and are eventually degraded.

The synaptic vesicle life cycle

Based on our data, we suggest the following sequence of events: synaptic vesicle precursors are produced in the soma, are transported to the synapse, where they are assembled into releasable vesicles. The vesicle proteins are used in exocytosis ~200 times during their lifecycle (Fig. 7c), and the protein assemblies get inactivated by contamination with SNAP25, which blocks CSPα in futile *cis*-complexes (Fig. 7d), before the vesicles are ultimately degraded.

Our results offer a new interpretation to the long-standing discussion on molecular differences between releasable and inactive “reserve” vesicles[11]. The difference between the two pools seems to be the age and usage of the vesicle molecules, measured by the accumulation of SNAP25 within their assemblies. Vesicles that work numerous times are

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identified by this mechanism, and are removed from the recycling pathway before they can become so badly damaged as to endanger the function of the synapse, and the organism's survival along with it. The inactive vesicles, which are reluctant to release and can only be forced to exocytose under strong supra-physiological stimulation, may not act as a reserve for neurotransmission, but are a collection of aged vesicles that are prevented from releasing. These vesicles may take on different roles in their late age, such as providing a buffer capacity for soluble co-factors of synaptic vesicle exo- and endocytosis[64].

SNAP25 and CSPα as molecular timer for synaptic vesicle inactivation

The role of SNAP25 in the SNARE complex that facilitates synaptic vesicle fusion to the cell membrane is well established[48], and CSPα has long been studied as a major chaperone involved in promoting the formation of the SNARE complex[50–52]. The knockout of SNAP25 leads to a complete failure of neurotransmission and death[65], as expected for such a critically important protein. The knockout of CSPα has much milder effects, albeit the mice develop neurological problems that lead to death within 1-2 months[66].

CSPα has been suggested to be mainly needed in priming SNAP25 for exocytosis, and presumably also in folding and stabilising this protein. Without CSPα, exocytosis upon single action potential stimulation is somewhat poorer, as would be expected from less efficient priming[51,54]. This phenotype is accompanied by a loss of SNAP25, since the stabilisation provided by CSPα is eliminated. Along the same lines, the overexpression of CSPα has been shown to help stabilize SNAP25, and to result in more synaptic vesicle fusion[52], as in our experiments (Fig. 7).

More unexpectedly, endocytosis is also poorer in mice lacking CSPα[54], which was difficult to ascribe to a role of CSPα in priming or stabilizing SNAP25. Our results provide a simple interpretation to this finding: older vesicles are more poorly retrieved (Supplementary Fig. 5c,d), which necessarily results in an endocytosis defect.

At the same time, these findings suggest that old vesicles are not inherently unable to release. They are only less able to do so than young vesicles, since they do not prime as efficiently as the young ones. When a young vesicle approaches the active zone, SNAP25 from the plasma membrane interacts with the CSPα from the vesicle surface, and is primed and readied for fusion. In contrast, for an old vesicle the CSPα molecules are less likely to prime the plasma membrane SNAP25, since they can alternatively interact with the vesicle-bound SNAP25. This makes such vesicles less able to prime, and probably prevents them from docking, as long as young vesicles are abundant in the vicinity. Especially under conditions of strong stimulation, however, where the young vesicles are all rapidly depleted, the aged vesicles could still be recruited to release, albeit with ever decreasing efficiency (Fig. 1d).

The inactivation of synaptic vesicles precedes the accumulation of damage to their proteins

Currently, there is little evidence available on how fast synaptic proteins might accumulate damage. However, if we assume that degradation of proteins occurs only after they have been damaged, the lifetimes of synaptic proteins in culture[31] can be used to predict the rates of damage to synaptic vesicle proteins (see Supplemental Experimental Procedures). We plotted the cumulative prediction of synaptic vesicle protein damage, and explored how much

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of the protein complement of one synaptic vesicle would be damaged at the time of inactivation (Supplementary Fig. 8). This calculation predicts that virtually no synaptic vesicle proteins are damaged at this time point, and that synaptic vesicles are removed just before the accumulation of damage begins. This indicates that the molecular timer we identified probably acts as a predictive mechanism, which pre-emptively removes vesicles from neurotransmission, before they can be damaged and become a hazard to cellular function, as outlined in the Introduction.

It is still unclear whether all membrane proteins of the aged vesicle will be degraded simultaneously in the cell body, or whether some, which are not yet damaged, will escape degradation. Such proteins could be again used in the formation of synaptic vesicles, as has been suggested in the past for dense-core vesicles[67], but this issue requires further investigation.

Conclusion

We conclude that the synapse evolved to accurately predict and pre-empt synaptic vesicle damage, using a molecular timer involving SNAP25 and CSPα. Synaptic vesicles are removed from activity just as they are expected to start accumulating damage. This is necessary since the cell depends on a fairly small population of releasable synaptic vesicles, which should not be compromised by even the slightest damage, if they are to ensure continued and reliable neurotransmission. We suggest that such mechanisms might be present in other cellular processes as well, especially if they depend on the action of only a few organelles at a time.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

S.O.R., S.T., and A.D. conceived the project. S.J. and A.V. (Leibniz Institute) performed the nanoSIMS experiments. E.F.F. performed the experiments on culture activity with GCaMP6 and sypHy. A.V. (Cells in Motion Cluster) performed the two-color STED experiments on changes in synaptic vesicle protein levels. S.T. performed all other experiments. H.W. developed the mathematical model of the synaptic vesicle life cycle. S.O.R., S.T., and E.F.F. analyzed the data. S.O.R. and S.T. prepared the manuscript.

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FIGURES AND FIGURE LEGENDS

Figure 1

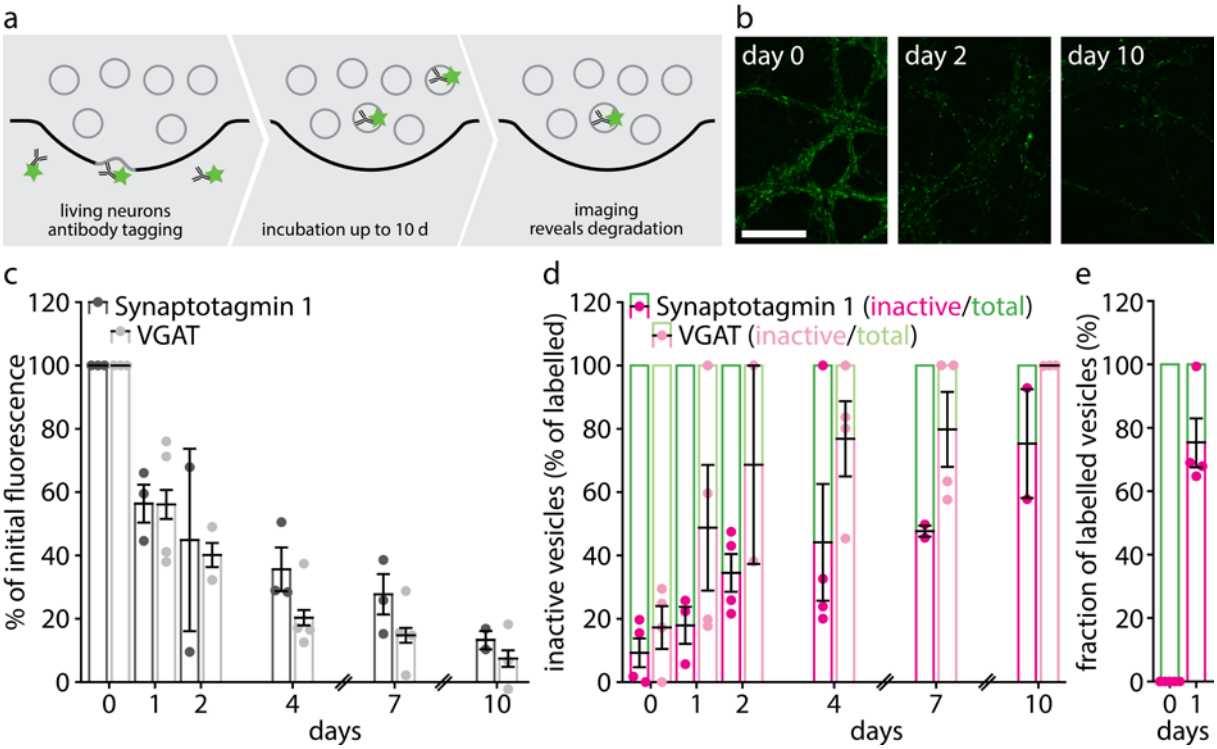

Figure 1: Ageing synaptic vesicle proteins stop releasing.

(a) To tag releasable synaptic vesicle proteins, we incubated the neurons with fluorophore-conjugated (green stars) antibodies against the lumenal domain of synaptic vesicle proteins, and imaged them up to 10 days later.

(b) Exemplary images of synapses labelled with synaptotagmin 1 antibodies. Scale bar: 20 µm.

(c) The loss of synaptic vesicle proteins from the synapse was monitored by imaging the antibody fluorescence at serial time points after tagging synaptotagmin 1 (dark grey; n = 3, 3, 2, 3, 3, 2 independent experiments per respective time point, at least 10 neurons sampled per experiment) or VGAT (light grey; n = 3, 4, 2, 4, 4, 3 independent experiments per respective time point, at least 10 neurons sampled per experiment).

(d) Synaptic vesicle responses to stimulation (20 Hz, 30 s), monitored by imaging antibodies conjugated to the pH-sensitive fluorophore CypHer5E. The antibodies were directed against synaptotagmin 1 (dark green and dark magenta; n = 4, 3, 4, 4, 2, 2 independent experiments per respective time point, at least 12 neurons sampled per experiment) or VGAT (light green and light magenta; n = 4, 4, 2, 4, 4, 3 independent experiments per respective time point, at least 8 neurons sampled per experiment). The VGAT follows the same approximate dynamic as synaptotagmin 1, but is faster. VGAT is present in only ~5-10% of the neurons in our cultures, and therefore this difference reflects a cell type-specific effect.

(e) A similar experiment, based on synaptotagmin 1 antibodies, was used to determine the fraction of vesicles of different ages that recycled during intrinsic network activity, in the absence of external stimulation (see Supplementary Fig. 3 for the protocol). Synaptotagmin 1 antibodies were preferred, since only a minority of the neurons in our cultures are VGAT-positive, and therefore they are not representative for the general spontaneous network

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activity of the cultures. The fraction of vesicle proteins that are still releasable under intrinsic network activity is only about half of that which can respond to high-frequency stimulation after 1 day. (n = 5, 4 independent experiments per respective time point, at least 10 neurons sampled per experiment).
All data represent the mean ± SEM.

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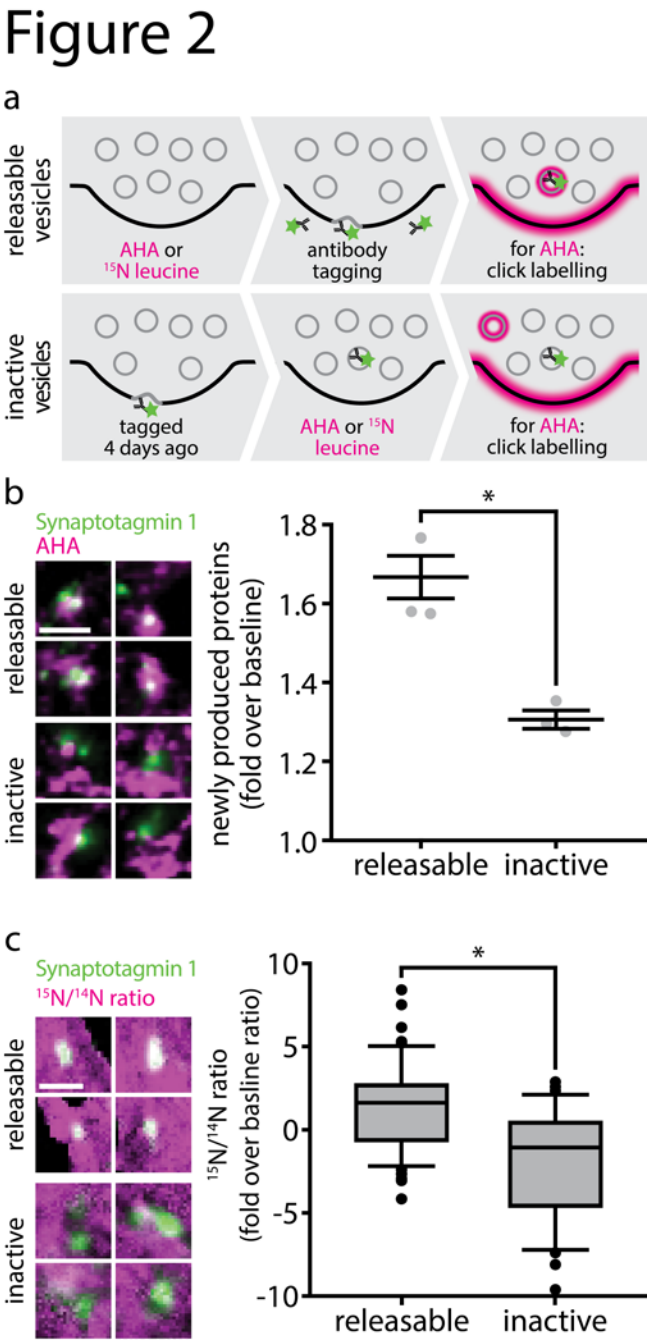

Figure 2: Metabolic imaging reveals that releasable synaptic vesicles are younger than inactive vesicles.

(a) To label newly produced proteins, we used the unnatural amino acid AHA for STED imaging, or ^{15}N-leucine for nanoSIMS imaging. These compounds were fed to neurons for 9 hours (AHA) or 24 hours (^{15}N-leucine) prior to the antibody tagging of releasable synaptic vesicles, after which we determined their co-localization with the antibodies. To compare the co-localization of young proteins with inactive synaptic vesicles, we performed the same experiment but with 3-4 days between antibody tagging and metabolic labelling.

(b) Co-localization of AHA with releasable and inactive synaptic vesicles in STED microscopy (n = 3 independent experiments per data point, at least 10 neurons sampled per experiment, *p = 0.0037; t(4) = 6.0980). Scale bar: 500 nm.

(c) ^{15}N/^{14}N ratio of releasable and inactive synaptic vesicles in combined fluorescence imaging and nanoSIMS (n = 57 synapses from 3 independent experiments for releasable

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synaptic vesicles, n = 47 synapses from 2 independent experiments for inactive vesicles, *p = 0.0001, t(102) = 5.5378). The ratio is a direct indication of the amount of ^{15}N-leucine in the vesicles. The inactive vesicles contain substantially fewer newly synthesized proteins than the rest of the axon (the ^{15}N/^{14}N ratio of which served as baseline; p = 0.0001, t(96) = 4.0691), while the releasable ones contain substantially more newly synthesized proteins (p = 0.0004, t(116) = 3.6156). Scale bar: 1 µm.
All data represent the mean ± SEM.

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Figure 3

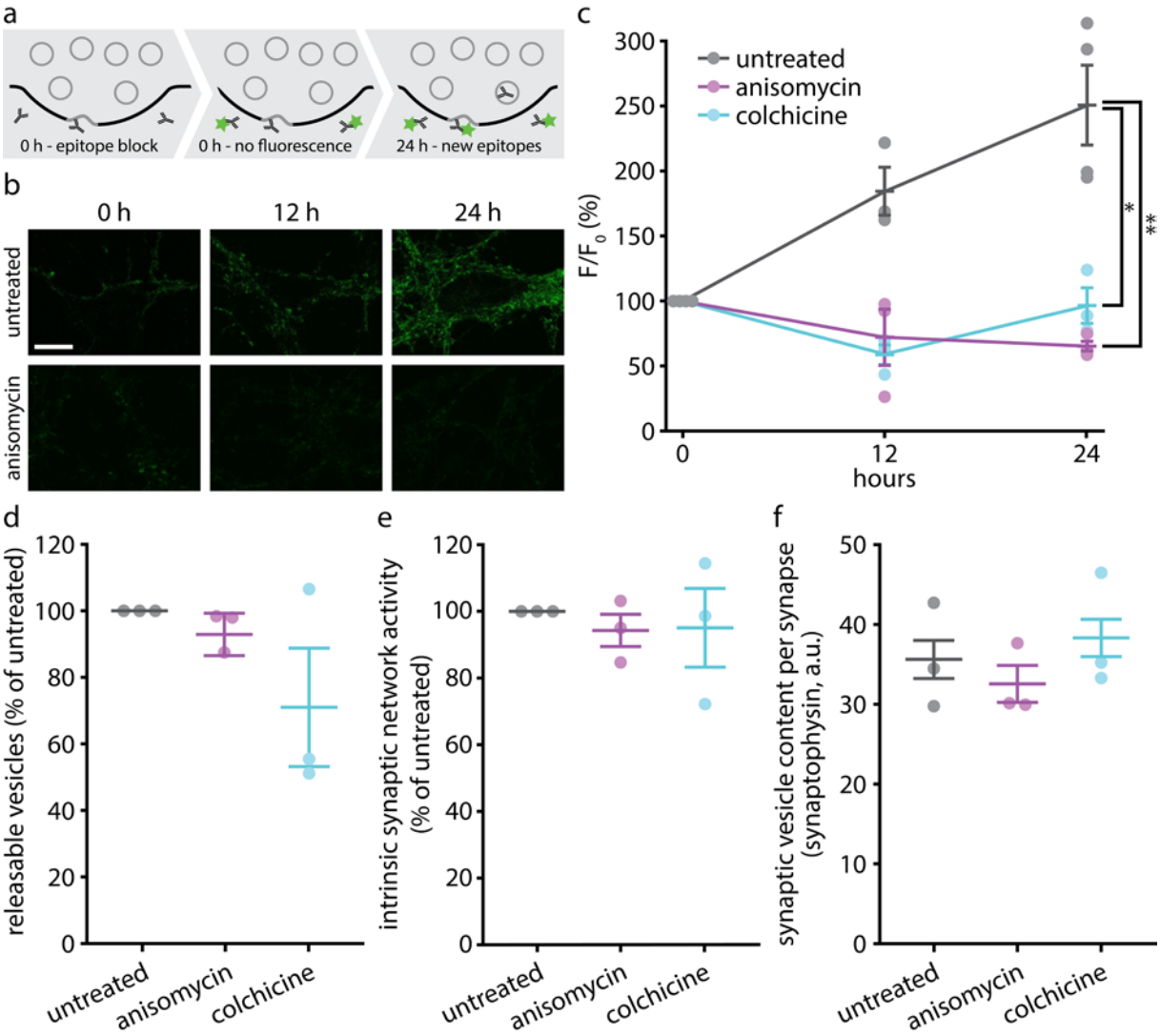

Figure 3: The synapse is dependent on young synaptic vesicles to turn over the active population.

(a) We saturated the lumenal synaptotagmin 1 epitopes on active synaptic vesicles with an unconjugated monoclonal antibody, and followed the replacement of the active population of vesicles by applying the same antibody, now fluorophore-conjugated, at serial time points.

(b) Exemplary images of replacement of the active population of synaptic vesicles. Scale bar: 10 µm.

(c) The active population of synaptic vesicles cannot be replaced when neurons are cut off from newly produced vesicles (anisomycin) or when vesicle transport from the soma to the synapse is disrupted (colchicine) (n = 4 independent experiments per data point for untreated 0 h and 24 h, n = 3 for all else, at least 10 neurons sampled per experiment; *p = 0.0104, t(5) = 3.9882; **p = 0.0041, t(5) = 5.0091).

(d) The drugs do not significantly impair synaptic activity (n = 3 independent experiments per data point, at least 10 neurons sampled per experiment; untreated vs. anisomycin, p = 0.2048, t(4) = 1.5131; untreated vs. colchicine, p = 0.1787, t(4) = 1.6286). Neurons were treated as in panel (a) for 24 h, to eliminate the entry of young vesicles into synapses, and were then stimulated in the presence of fluorescently conjugated synaptotagmin 1 antibodies (20 Hz, 30 s). The fluorescence intensity was recorded as a measure of antibody uptake into

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recycling vesicles. Only a limited decrease in recycling was observed in drug-treated preparations.

(e) Drug effects on the intrinsic network activity. To assess the intrinsic network activity of our cultures after 24 h of drug treatment, we measured vesicle recycling during the last hour of the treatment. The network activity did not change significantly (n = 3 independent experiments per data point, at least 10 neurons sampled per experiment; untreated vs. anisomycin, $p = 0.3317$, $t(4) = 1.1035$; untreated vs. colchicine, $p = 0.6998$, $t(4) = 0.4144$).

(f) The total synaptic vesicle pool size after 24 h of drug treatment. This value was determined by immunostaining for a major synaptic vesicle marker, synaptophysin. There were no significant changes in synaptic vesicle pool size (n = 3 independent experiments per data point, at least 10 neurons sampled per experiment; untreated vs. anisomycin, $p = 0.5374$, $t(4) = 0.6738$; untreated vs. colchicine, $p = 0.6566$, $t(4) = 0.4796$).

All data represent the mean ± SEM.

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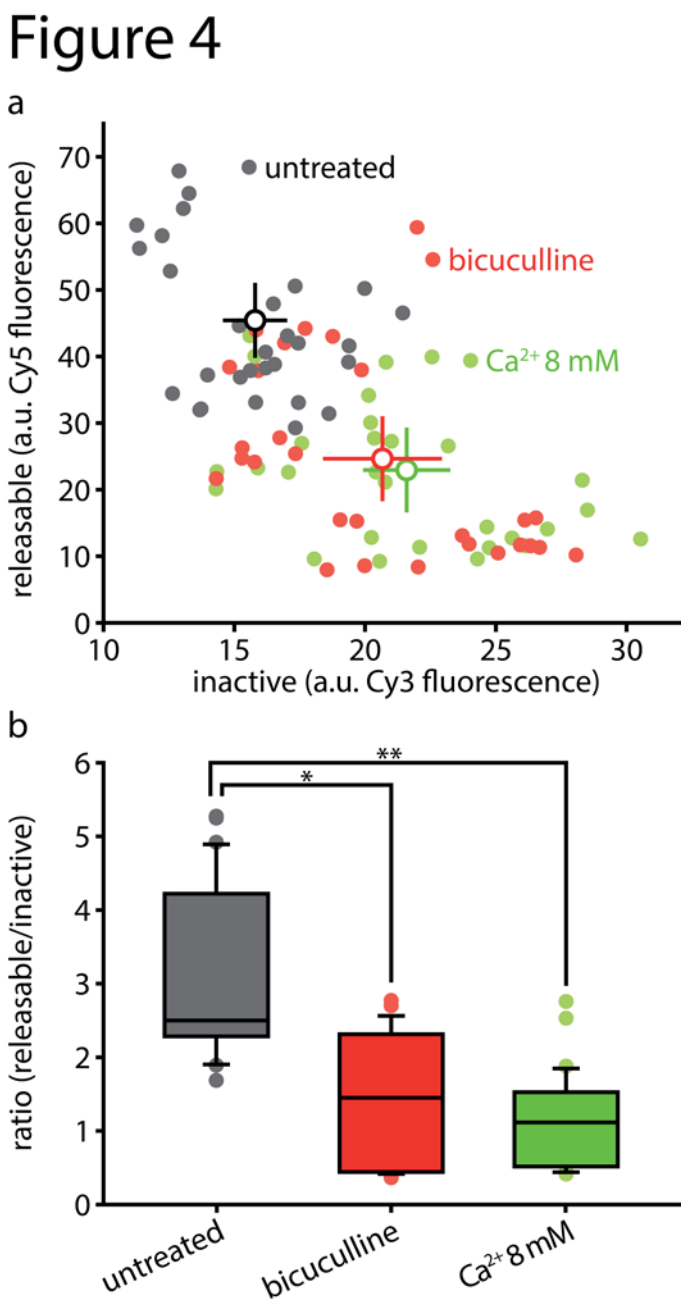

Figure 4: Increased synaptic activity accelerates synaptic vesicle ageing.
(a) Releasable and inactive vesicles were tagged after 12 h of incubation in bicuculline or 8 mM Ca^{2+} to increase synaptic activity, or with untreated medium as a control (n = 30 neurons for untreated, 29 neurons for bicuculline, 30 neurons for Ca^{2+} 8mM, from 3 independent experiments per condition). The intensity of the signal ascribed to releasable or inactive vesicles is shown.
(b) Ratio of releasable vs. inactive vesicles, from (a); *p = 0.0001, t(57) = 6.2692; **p = 0.0001, t(58) = 7.8419.
All data represent the mean ± SEM.

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Figure 5

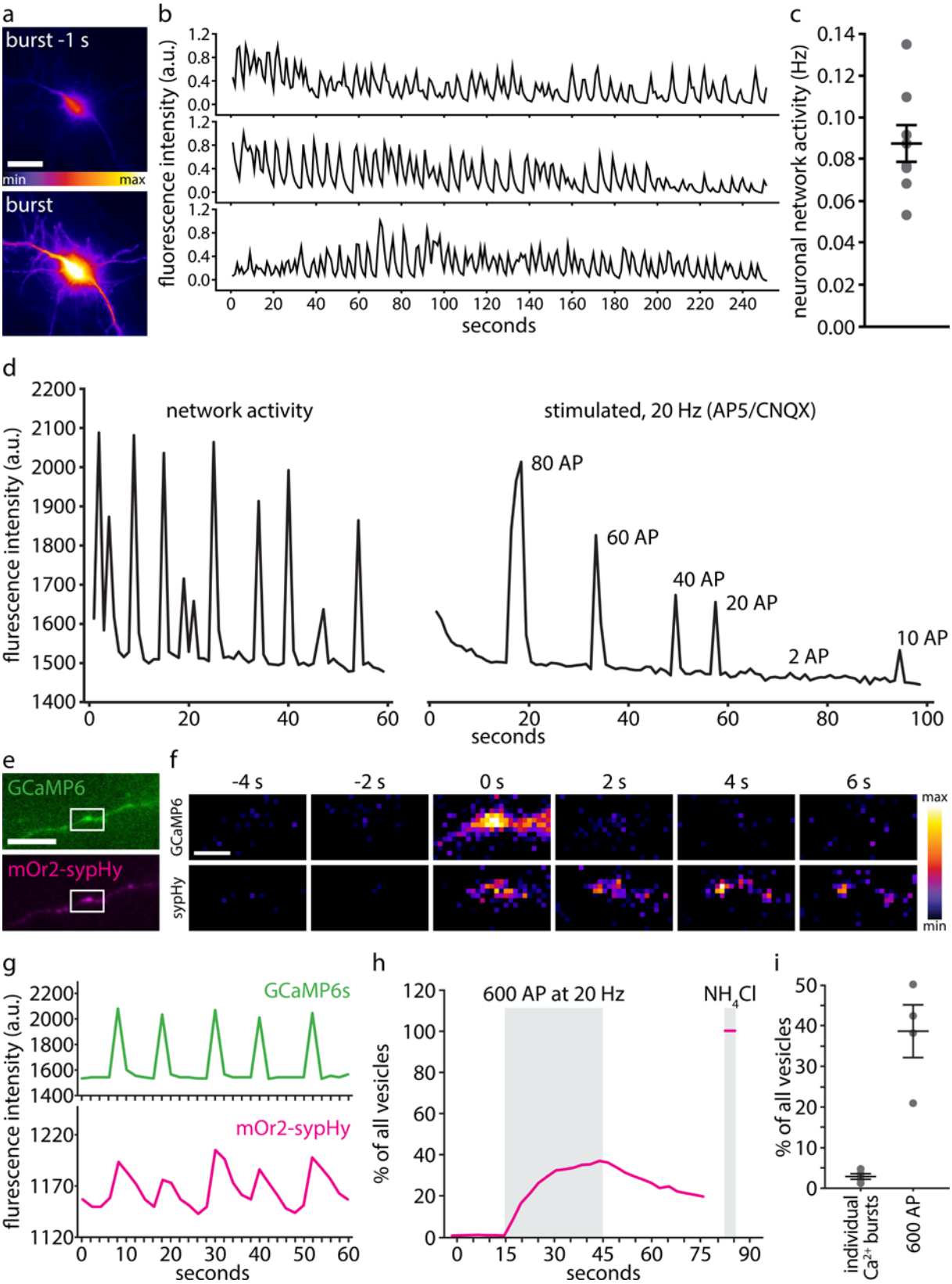

Figure 5: An analysis of the synaptic vesicle recycling under intrinsic network activity. (a) Exemplary images of a neuron transfected with GCaMP6 to assess the frequency of activity in culture. Scale bar 20 µm.

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(b) Exemplary traces of intrinsic network activity in hippocampal cultures.
(c) Average activity frequency in hippocampal cultures.
(d) A comparison of the Ca^{2+} signals induced by network activity bursts (left) and 20 Hz trains of different lengths (right). This suggests that the bursts consist of several tens of action potentials.
(e) Exemplary images of the synapse of a neuron co-transfected with GCaMP6 and synaptopHluorin, to assess the levels of vesicle release coinciding with activity bursts. Scale bar 2 µm.
(f) Exemplary images of changes in GCaMP6 and sypHy fluorescence during a pulse of intrinsic network activity (white box magnified from (e)).
(g) Exemplary traces of activity and release levels in hippocampal cultures.
(Hh) An exemplary trace of the release of vesicles during a train of electrical stimulation with 600 action potentials (AP) at 20 Hz, as measured by changes in sypHy fluorescence. To assess the total pool of vesicles and scale the releasing fraction accordingly, a pulse of NH_4Cl was applied at the end of the experiment.
(i) Quantification of the fraction of synaptic vesicle proteins exocytosed during one activity burst.

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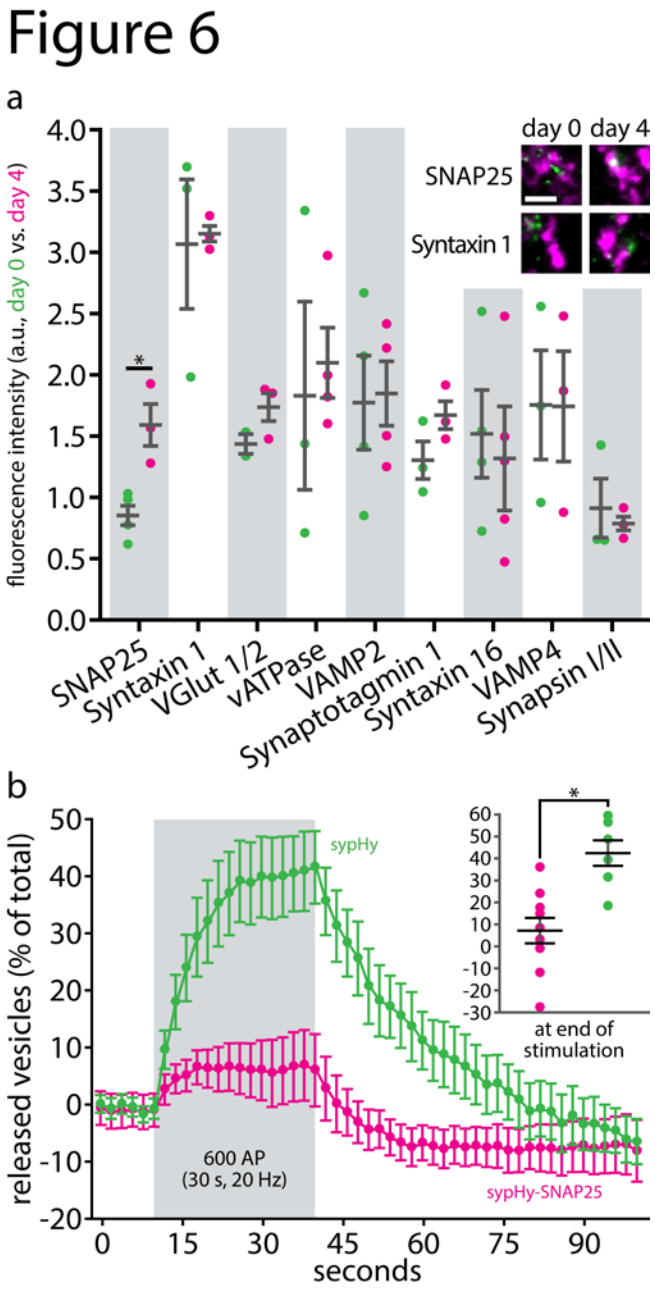

Figure 6: SNAP25 enters ageing synaptic vesicles and inactivates them.
(a) Two-color STED analysis of changes in synaptic vesicle protein levels during the transition from the releasable state to the inactive state. Living neurons were labelled with synaptotagmin 1 lumenal antibodies and then co-immunostained for the proteins of interest either directly, for association with the young, active population, or 3-4 days later, for association with the old, inactive population. We selected abundant and functionally important proteins of interest from every compartment the vesicle passes through during recycling (synaptic vesicle, endosome, cell membrane). Before imaging, the samples were embedded in melamine and cut into 50 nm thin sections to achieve single vesicle resolution in all dimensions. Scale bar 500 nm. The only protein whose levels change significantly is SNAP25 (SNAP25, n (day 0) = 4, n (day 4) = 3, *p = 0.0124, t(5) = 3.8200; Syntaxin 1, n (day 0) = 3, n (day 4) = 3, p = 0.8850, t(4) = 0.1541; VGlut 1/2, n (day 0) = 2, n (day 4) = 3, p = 0.1986, t(3) = 1.6447; vATPase, n (day 0) = 3, n (day 4) = 4, p = 0.7340, t(5) = 0.3594; VAMP2, n (day 0) = 4, n (day 4) = 4, p = 0.8837, t(6) = 0.1527; Synaptotagmin 1, n (day 0) =

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3, n (day 4) = 3, p = 0.1604, t(4) = 1.7208; Syntaxin 16, n (day 0) = 4, n (day 4) = 4, p = 0.7406, t(6) = 0.3468; VAMP4, n (day 0) = 3, n (day 4) = 3, p = 0.9863, t(4) = 0.0183; Synapsin I/II, n (day 0) = 3, n (day 4) = 3, p = 0.6638, t(4) = 0.4685; at least 10 neurons sampled per experiment).

(b) Synaptic vesicles carrying a sypHy construct respond normally to stimulation, while synaptic vesicles carrying a sypHy-SNAP25 construct to target SNAP25 specifically to synaptic vesicles display a strongly reduced response (n = 6 independent experiments for sypHy, n = 9 independent experiments for sypHy-SNAP25; *p = 0.0025, t(13) = 3.7334). Cells were stimulated with 600 action potentials at 20 Hz to trigger the release of all releasable synaptic vesicles, 3-4 days after transfection. NH_4Cl was used to reveal all vesicle epitopes, to scale the released fraction.

All data represent the mean ± SEM.

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Figure 7

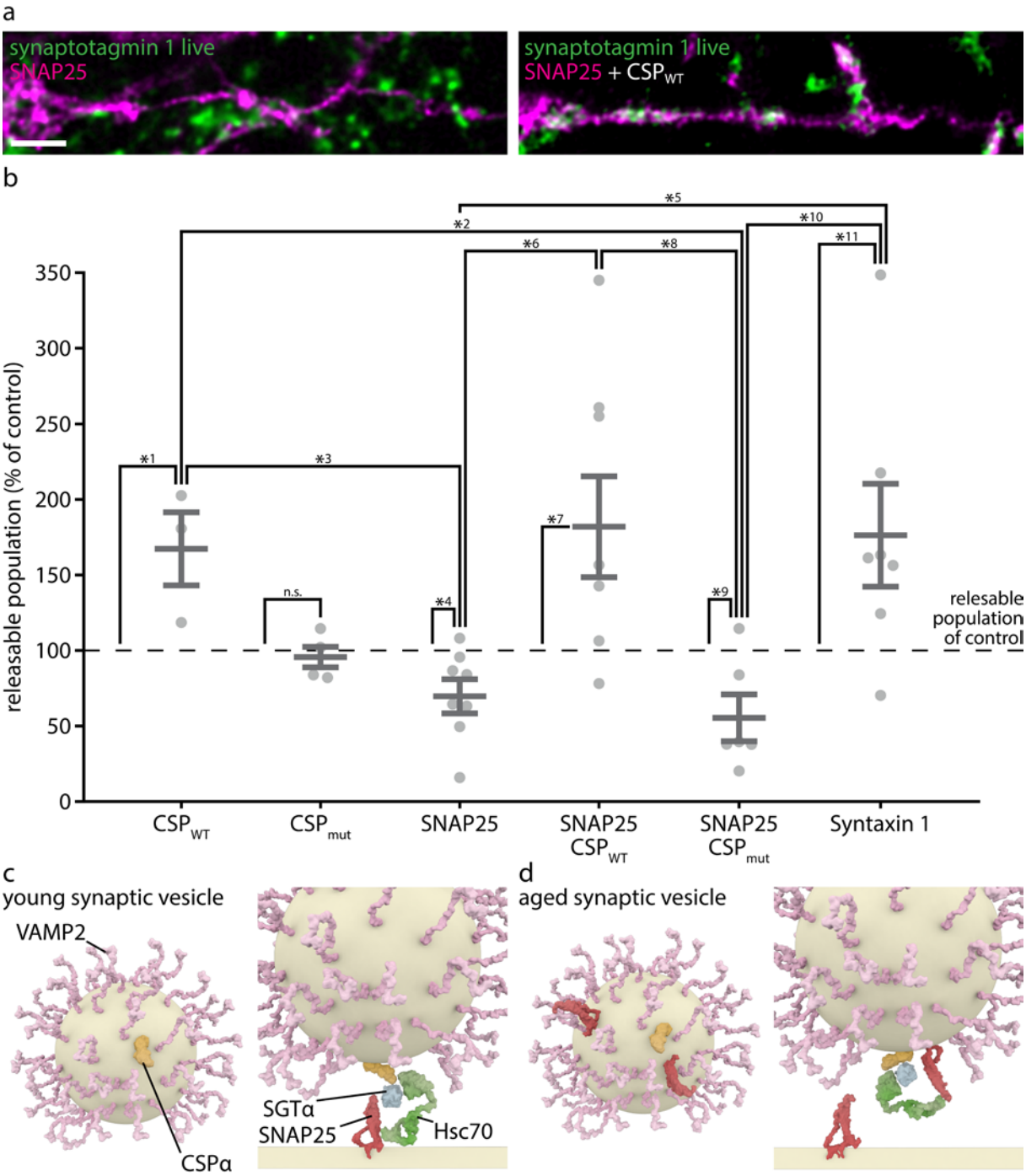

Figure 7: SNAP25 inactivates synaptic vesicles by blocking CSPα on the vesicle.
(a) Exemplary images showing reduced vesicle recycling in neurons overexpressing SNAP25 (upper panel), compared to neurons overexpressing both SNAP25 and wild-type CSPα (CSP_{WT}). Scale bar: 2 µm.
(b) Quantification of levels of release in neurons overexpressing CSP_{WT} (n = 16 transfected neurons from 3 independent experiments), a mutated version of CSPα unable to target to vesicles and thus incapable of interacting with SNAP25[51] (CSP_{mut}, n = 11 transfected neurons from 4 independent experiments), SNAP25 (n = 24 transfected neurons from 8 independent experiments), SNAP 25 + CSP_{WT} (n = 20 transfected neurons from 8 independent experiments), SNAP25 + CSP_{mut} (n = 20 transfected neurons from 6

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independent experiments), and, as a control, syntaxin 1 (n = 13 transfected neurons from 7 independent experiments). The lower dashed line represents the normal level of activity; the upper dashed line indicates the total vesicle pool at the synapse (see Supplementary Fig. 2c). The significance levels determined are: $*^{1}p = 0.0478$, $t(4) = 2.8201$; $*^{2}p = 0.0039$, $t(7) = 4.2307$; $*^{3}p = 0.0017$, $t(9) = 4.4222$; $*^{4}p = 0.0139$, $t(14) = 2.8091$; $*^{5}p = 0.0064$, $t(13) = 3.2479$; $*^{6}p = 0.0049$, $t(13) = 3.3869$; $*^{7}p = 0.0269$, $t(12) = 2.516$; $*^{8}p = 0.0077$, $t(11) = 3.2530$; $*^{9}p = 0.0126$, $t(10) = 3.0354$; $*^{10}p = 0.0090$, $t(11) = 3.1659$; $*^{11}p = 0.0378$, $t(12) = 2.3346$; $^{n.s.}p = 0.5916$, $t(6) = 0.5664$.

(c) SNAP25 and CSPα interact with SGTα and Hsc70 in *trans*, promoting synaptic vesicle fusion[51].

(d) SNAP25 on the aged vesicles may interact in *cis* with CSPα, thus sequestering it from its *trans* interaction with SNAP25 molecules from the plasma membrane.

All data represent the mean ± SEM.