A small pool of vesicles maintains synaptic activity in vivo

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Chemical synapses contain substantial numbers of neurotransmitter-filled synaptic vesicles, ranging from approximately 100 to many thousands. The vesicles fuse with the plasma membrane to release neurotransmitter and are subsequently reformed and recycled. Stimulation of synapses in vitro generally causes the majority of the synaptic vesicles to release neurotransmitter, leading to the assumption that synapses contain numerous vesicles to sustain transmission during high activity. We tested this assumption by an approach we termed cellular ethology, monitoring vesicle function in behaving animals (10 animal models, nematodes to mammals). Using FM dye photooxidation, pHluorin imaging, and HRP uptake we found that only approximately 1–5% of the vesicles recycled over several hours, in both CNS synapses and neuromuscular junctions. These vesicles recycle repeatedly, intermixing slowly (over hours) with the reserve vesicles. The latter can eventually release when recycling is inhibited in vivo but do not seem to participate under normal activity. Vesicle recycling increased only to ≈5% in animals subjected to an extreme stress situation (frog predation on locusts). Synapsin, a molecule binding both vesicles and the cytoskeleton, may be a marker for the reserve vesicles: the proportion of vesicles recycling in vivo increased to 30% in synapsin-null *Drosophila*. We conclude that synapses do not require numerous reserve vesicles to sustain neurotransmitter release and thus may use them for other purposes, examined in the accompanying paper.

pools | styryl dyes | vesicle cycle | shibire | calyx of Held

Upon neuronal activity, synaptic vesicles fuse with the plasma membrane and release their neurotransmitter contents. The vesicles then reform and are refilled with neurotransmitter, in what is termed vesicle recycling (reviewed in ref. 1). Many vesicles can be contained within a synapse, with at least ≈500,000, for example, in the well-studied frog neuromuscular junction (NMJ) (2). Generally, synaptic release sites are faced by ≈200–300 vesicles, with less than one vesicle being released per action potential (2). It has been assumed for many decades that the sole function of all vesicles is to release neurotransmitter—a logical assumption in view of the fact that they all seem to be filled with neurotransmitter molecules. In agreement with this hypothesis, it has been demonstrated countless times that all vesicles can be forced to release when preparations are stimulated in vitro at relatively high frequencies (10–100 Hz, for many seconds or minutes). Unsurprisingly, milder stimulation causes the recycling of substantially fewer vesicles, a fact most evident in mammalian CNS preparations kept at room temperature [which release only ≈20–50% of the vesicles over a few minutes of stimulation (3–5); up to 100% for ref. 6]. The remaining vesicles constitute what has been defined as the "reserve pool," a reservoir of vesicles currently thought to be needed upon high synaptic activity (2).

The in vivo situation is still a matter of conjecture. High levels of activity can be attained in living animals (even in the range of several hundred Hz; for example, ref. 7), albeit only for brief periods of time. At least for the NMJs, postsynaptic function places an upper limit on vesicle use in vivo: the prolonged high-frequency activity required to release the reserve pool in vitro is useless for the living animal, because the muscle rapidly ceases to respond (reviewed, for example, in ref. 8). The recent discovery that modulation of cyclin-dependent kinase 5 can change the activity of the reserve pool in vitro (9) renders the question of whether these vesicles are used in vivo even more pressing.

Although experimental data are lacking, reasonable estimates of in vivo release can be gathered for some well-studied synapses. For example, at the frog NMJ single action potentials are sufficient for muscle contractions in vitro, and one action potential causes the release of ≈100 vesicles [up to, at most, ≈400 vesicles (10)]. With a recycling time of approximately 60 s (11), this synapse could easily sustain physiological frequencies of activity of 1–2 Hz using only 5–10% of its vesicles. In CNS synapses the number of vesicles is much smaller [≈200 vesicles in a synaptic bouton (2)], but the release probability of the vesicles is also low (for example, ref. 12), again resulting in limited vesicle use. As a further example, the calyx of Held from the auditory pathway is normally active at ≈30 Hz in vivo (13) and can sustain activity also at much higher rates (up to ≈600 Hz). To cope with such activity, the calyx of Held is a very large synapse, containing hundreds of active zones [≈550 (14)] and hundreds of thousands of vesicles (4). However, because this synapse has a rather small quantal content in vivo [fewer than 20 vesicles (15)], very few of its active zones would release a vesicle at any one time during 30-Hz firing. Assuming a recycling rate of 60 s (as above), 20% of its vesicles would still be sufficient for normal calyx function (although, of course, substantially more vesicles would recycle under 600-Hz activity).

Thus, synaptic activity in vivo may actually not need to use all that many vesicles. To examine this issue, we decided to study synaptic function under physiological conditions, in an approach we define as cellular ethology—the observation of the natural behavior of cells. Although admittedly less precise than experimental manipulation, we see the ethological approach as much more relevant to the cell/organism behavior. We found that only a small fraction of vesicles is used repeatedly during physiological

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activity, whereas the large majority of the vesicles do not undergo exocytosis and recycling, even during extreme stress.

Results

Few Synaptic Vesicles Are Used for Neurotransmitter Release. Our first approach was to inject the fluorescent dye FM 1-43 into several animal species (either in the body cavity or s.c.) and afterward allow the animals to move around freely for a specified amount of time (from 10 min to 4 h), during which the dye was taken up by recycling vesicles (Fig. 1*A*) (*Methods*). The organs containing the synapses of interest were then rapidly dissected, fixed, and illuminated in the presence of di-amino-benzidine (DAB). This induces DAB to precipitate within the dye-labeled vesicles (photooxidation), which are then counted by electron microscopy (18–20).

We first focused on NMJ preparations (Fig. 1*B*). We used body motion muscles in *Drosophila* larvae and in the nematode worm *Caenorhabditis elegans*, leg muscles in locust (*Chorthippus biguttulus*), tail muscles in zebrafish (*Danio rerio*), the *cutaneous pectoris* chest muscle in frog (*Rana kl. esculenta*), and the ear-lifting muscle *levator auris longus* in mouse (*Mus musculus*). Surprisingly, in all of these preparations only 1–5% of the vesicles were labeled in vivo over several hours (Fig. 1 *B* and *C*), with the labeling increasing slowly, at 1% to 2% per hour for the *Drosophila* or frog NMJs (Fig. S1*A*). As expected, substantially more vesicles could be labeled upon in vitro stimulation (Fig. S1*B*).

The same finding was obtained in developing synapses (leg muscles in chicken embryos, *Gallus gallus*), in CNS synapses involved in motor control (in the *Drosophila* ventral ganglia), and in CNS synapses from a sensory pathway (in the optic lobe of the cricket, *Gryllus assimilis*; Fig. 1 *B* and *C*). Finally, we also studied the calyx of Held synapse in the rat auditory pathway, because it is known to be highly active in vivo (see above). To avoid difficulties with FM photooxidation deep within brain slices, we stereotaxically injected into this preparation HRP, which is taken up well into synaptic vesicles (21). The HRP-containing vesicles were identified by DAB oxidation and provided a similar result: few vesicles were labeled (Fig. 1 *B* and *C*).

Clearly, labeling of only a small proportion of vesicles could result from various problems with the technique. However, a number of control experiments rule out such an explanation: the dye was present in the body fluids for hours (Fig. S2), the number of electrophysiologically detected quanta was identical, within experimental error, to the number of vesicles labeled by FM photooxidation (Fig. S3; note that this experiment was conducted in vitro), and the dye was fully available for vesicle uptake (Fig. S4). Additionally, other cells, such as the Schwann cells of the frog (Fig. S5), took up the dye in substantial amounts in vivo, again confirming the efficiency of the procedure. The labeled and nonlabeled vesicles were easily distinguished in all preparations (Fig. S5 *A*–*C*). Finally, we note that photooxidation can label essentially all vesicles, as we have shown in the past (see Supplementary Figure 2 of reference 20), and strong stimulation will label the large majority of the vesicles at least in some preparations (Fig. S4 *D* and *E*).

PHluorin Imaging Confirms the Limited Vesicle Use in Vivo. To verify these unexpected findings by an independent technique, we turned to fluorescence imaging of *Drosophila* larvae expressing a pH-sensitive GFP moiety (pHluorin) linked to the intravesicular end of synaptobrevin (22, 23). The pH-sensitive GFP is quenched in the acidic interior of the vesicles and is only fluorescent when the vesicles fuse to the plasma membrane. The pHluorin fluorescence was low in living larvae, indicating that only a handful of vesicles are recycling at any one time (Fig. 2 *A* and *B*).

This experiment has a rather limited precision, giving only a broad overview of recycling in vivo. It is also unable to quantify the number of vesicles recycling over a longer period. To test this, we used an inhibitor of vesicle acidification, bafilomycin (24, 25). Vesicles retrieved from the plasma membrane are not able to reacidify in the presence of bafilomycin and remain fluorescent (Fig. 2*C*). We injected bafilomycin (≈1 μM) in living larvae and allowed them to behave for 10–120 min, before dissection and monitoring by fluorescence microscopy (see model in Fig. 2*D*). The larvae lost coordinated movement within ≈2 min after injection, becoming largely paralyzed. Some movement was nevertheless observed, especially during the added stress of pinning and dissection, which indicates that the upstream neurons could still fire and trigger occasional muscle responses (likely because the low amount of bafilomycin we used penetrates faster into the NMJs than into the compact brain of the animal). The fluorescence of the preparations did not increase significantly after bafilomycin injection ($P > 0.1$, t test, $n = 4$–9 experiments), indicating that only a few vesicles are used in vivo. Because the fluorescence of different preparations is to some extent variable (owing to different levels of pHluorin expression), we also measured the fractional increase in fluorescence upon NH_4Cl addition. This treatment neutralizes the vesicular pH and therefore reports the size of the vesicle pool that did not recycle during the bafilomycin treatment. The size of this pool was indistinguishable from that of control (noninjected) preparations (Fig. 2 *E* and *F*). Again, we conclude that only a minor fraction of the vesicles recycle in vivo over many minutes/hours.

Importantly, the results obtained with FM 1-43 or HRP (Fig. 1) are to some extent flawed by the possibility that vesicles release neurotransmitter through small or short-lived pores (kiss-and-run) without becoming labeled (i.e., without allowing the entry of the label). This is no longer a concern for pHluorin imaging, whereby signal intensity depends only on the release of protons from the vesicles. Thus, it is unlikely that a high incidence of kiss-and-run release in vivo would bias our findings.

Electron Microscopy in *shibire* Fruit Flies Is Compatible with These Findings. To verify this conclusion by an assay in which no injection of dye or drugs is performed, we turned to the temperature-sensitive *Drosophila* dynamin mutant (*shibire*). Larvae placed at the nonpermissive temperature paralyzed within 10–15 s (Fig. S6*A*), a time point at which their synapses still contained numerous vesicles—indistinguishable from controls kept at permissive temperature (Fig. S6 *B* and *C*). Two explanations can be offered for this phenotype: first, the loss of a small pool of vesicles may be sufficient to block effective neurotransmission in vivo; second, as the endocytic machinery is inhibited, the release site is still occupied by fused vesicle molecules, which slows the release of subsequent vesicles (26; reviewed in ref. 27). Although it is difficult to differentiate between these possibilities, we conclude that the behavior of the *shibire* larvae is compatible with our hypothesis. More importantly, the remaining vesicles were eventually released, with ≈60% depleted at 10 min after the switch to nonpermissive temperature (Fig. S6 *B* and *C*). This allows the conclusion that the switch from inactive to active vesicles must be a relatively simple one, with the loss of the "active" vesicles (due to recycling inhibition) triggering the activation of the reserve vesicles (see also below). This finding was not restricted to the larvae: adult flies behaved in an identical fashion (see analysis of a leg muscle synapse in Fig. S6 *D*–*F*).

Importantly, the strong vesicle depletion over 10 min indicates that numerous vesicle release events do occur in vivo, or in other words that activity in vivo is substantial. Therefore, coupling this also with the results from our FM photooxidation and bafilomycin/pHluorin experiments, we reach the conclusion that the few vesicles that recycle in vivo must do so repeatedly, constantly recycling and rereleasing.

Few Synaptic Vesicles Are Used for Neurotransmission Even in a Stress Situation. It could nevertheless be argued that more vesicles would be used upon higher in vivo activity. In laboratory settings, ex-

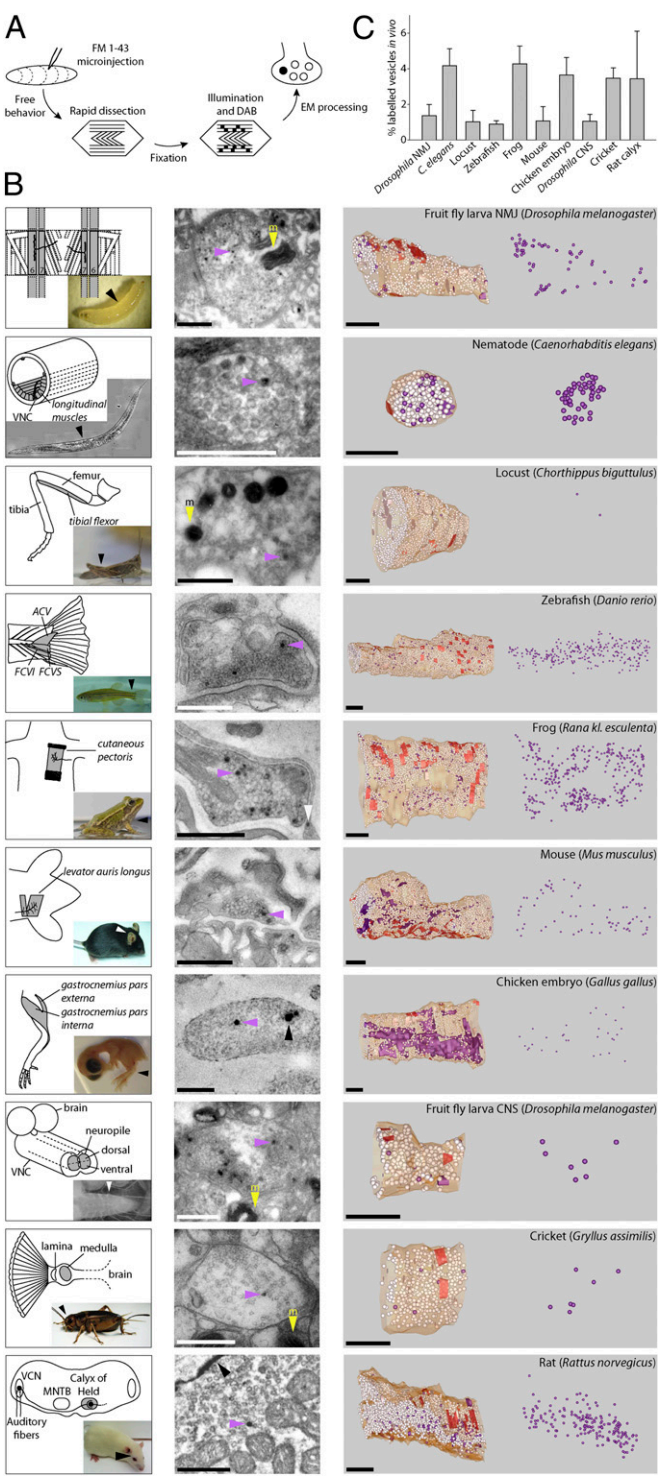

Fig. 1. Only a small percentage of the synaptic vesicles undergo exocytosis and recycling in vivo. (*A*) General schematic of the photooxidation experiments (see text for details). (*B*) Typical examples. For all preparations, *Left* depicts the organs of interest (gray) and their approximate position within the animal (arrowheads). *Middle* shows representative electron micrographs. Purple arrowheads indicate example labeled vesicles. Yellow arrowheads indicate DAB-labeled mitochondria; their labeling is independent of FM staining (16). White arrowhead in the frog panel indicates an endosomal structure labeled in the Schwann cell. Black arrowhead in the chicken panel indicates a labeled vacuole (note vacuoles in 3D reconstruction as well). *Right* shows 3D reconstructions of nerve terminals (rightmost drawing shows only labeled vesicles). The plasma membrane is yellow; intraterminal membranes (vesicles and vacuoles) are purple if labeled and white if unlabeled; active zones are red. Species name is indicated in the upper right corner. VNC, ventral nerve cord; ACV, *adductor caudalis ventralis*; FCVI, *flexor caudalis ventralis inferior*; FCVS, *flexor caudalis ventralis superior*; VCN, ventral cochlear nucleus; MNTB, medial nucleus of the trapezoid body. Note the presence of only a few labeled vesicles in all preparations. HRP uptake, rather than FM 1-43 uptake, was used for the calyx of Held; black arrowhead in the calyx of Held panel indicates HRP outside of the cell. The zebrafish scheme was drawn after ref. 17. (Scale bars, 500 nm.) (*C*) Percentage of labeled (dark) vesicles in vivo at 2 h after injection (mean ± SEM from at least four independent preparations from each species; mean ± range of values for the calyx of Held, for which the value at 30 min after injection is shown; similar values were obtained at 60 min after injection).

perimental animals live in a low-stress environment with reduced stimuli, and it may be argued that our in vivo experimental conditions fail to cover extreme life situations associated with high stress levels. We therefore sought to reproduce a setting in which one organism is exposed to maximal stress—while still in a natural context, remaining within the boundaries of a cellular ethology approach, rather than resorting to artificial stimuli.

We took advantage of the fact that the *tibial flexor* muscle of the third pair of legs of the locust is the main muscle involved in the animal's escape mechanism (jumping). We injected locusts as above, and after 2 h we placed them individually in a terrarium occupied by several frogs (i.e., natural predators of locusts). The locusts avoided the frogs actively, with frequent jumping (Fig. 3*A*). After numerous failed attempts (over ≈5–10 min) each locust was caught and eventually ingested (Fig. 3*A*, *Right*). At this moment the frog was sacrificed and the locust retrieved and its muscle photooxidized. Surprisingly, even after such extreme stress only 5% of all synaptic vesicles were labeled (Fig. 3 *B–D*), increasing from ≈1% in the nonstressed animal. We note that the prolonged struggle caused by restricting movement to a small terrarium was more stressful for the locust than a comparable situation in the natural habitat, in which the locust is either caught during the first attempt or else escapes after the initial jumps (with the distance covered by a locust jump being substantially beyond the range of the frogs).

Synapsin Differentiates Between the Active and Inactive Vesicles. From the experiments presented above, it is easy to hypothesize that a physical barrier may inhibit the release of the majority of the vesicles, perhaps by lowering their mobility. Synapsin has been proposed to bind to synaptic vesicles and/or to the cytoskeleton, thus acting as a "glue" keeping vesicles in a clustered (possibly inactive) state (see ref. 28). To test whether vesicle clustering may affect their release in vivo, we studied synapsin-null *Drosophila* larvae (29). Indeed, fluorescence recovery after photobleaching (FRAP) experiments revealed that the vesicles were significantly more mobile in synapsin-null animals [Fig. 4 *A* and *B*; note that the recovery observed also in the wild type is largely due to the recovery of the background fluorescence (30)]. The vesicle labeling was also much stronger in vivo, with approximately 30% of the vesicles labeled at 2–4 h after injection (Fig. 4 *C* and *D*), allowing the conclusion that synapsin is one potential marker for the inactive vesicles.

Because synapsin is not the only molecule cross-linking vesicles (31), it is not unexpected that a substantial nonreleasing pool of vesicles persists in the synapsin-null animals. Importantly, synapsin is a soluble molecule, rather than an integral component of the vesicles, and therefore it is not likely to constitute a permanent vesicle tag. Thus, an intermixing of the active and inactive vesicles should take place over time, although it seems to be on a very slow scale, of a few hours (Fig. S1*A*).

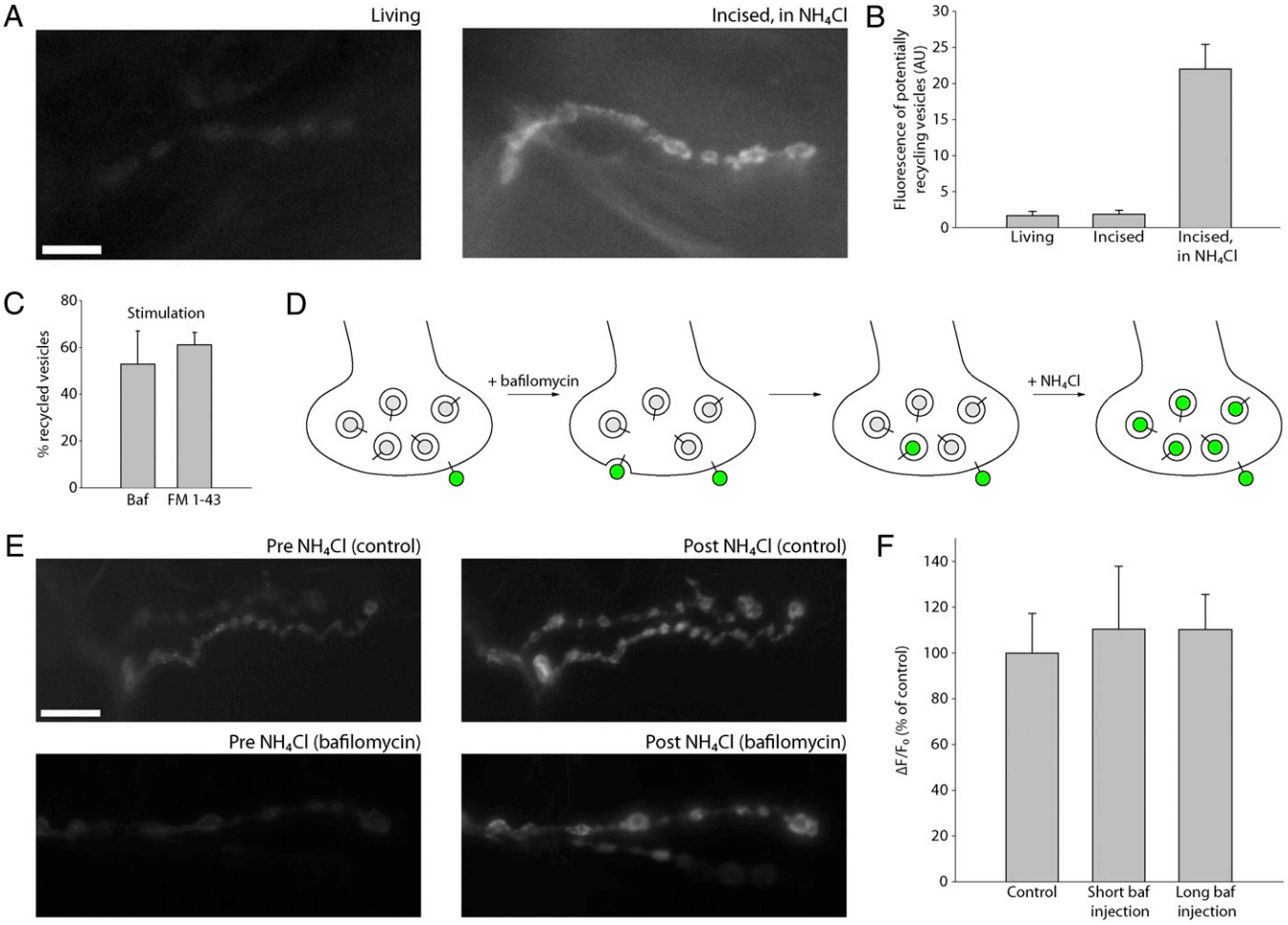

Fig. 2. PHluorin imaging confirms that few synaptic vesicles recycle in the *Drosophila melanogaster* larval NMJ in vivo. (*A*) Living larvae were pinned ventral side up, and the fluorescence of individual synapses was then imaged through the cuticula (*Left*). To detect the fluorescence of the resting (quenched) vesicles we performed lateral incisions in the cuticula and imaged the preparations in presence of 100 mM NH_4Cl, which neutralized the vesicular pH (*Right*). (Scale bar, 10 μm.) (*B*) Only a small amount of fluorescence is detectable in the living larvae; the lateral incision itself does not increase this amount, whereas NH_4Cl reveals a very large pool of resting vesicles (means ± SEM from five experiments). (*C*) To test the effects of bafilomycin in the *Drosophila* NMJ, we stimulated dissected pHluorin larvae at 30 Hz, for 5 min in its presence. The fluorescence increase obtained in the presence of bafilomycin was expressed as percentage of the increase obtained upon application of NH_4Cl (i.e., as percentage of all vesicles) (mean ± SEM from five independent experiments). The percentage of vesicles labeled with FM 1-43 under identical stimulation conditions is reproduced from Fig. S1*B*, for comparison. Note that the two approaches correlate closely. (*D*) Experimental approach devised to quantify the amount of vesicles recycling over a defined time period in vivo. Bafilomycin was injected in the animals; it blocks vesicle acidification, and therefore vesicles recycling in presence of bafilomycin remain fluorescent (*Middle*). At the end of the experiment the larvae are dissected and imaged, and NH_4Cl is applied to reveal the remaining pool of unused vesicles. (*E*) Typical images of control or bafilomycin-injected larvae, before and after NH_4Cl application. (Scale bar, 10 μm.) (*F*) Quantification of the size of the unused vesicle pool. The fractional fluorescence increase upon NH_4Cl application is expressed as percentage of control, uninjected larvae (means ± SEM from four to nine independent experiments). Short injection: 10–30 min. Long injection: 120 min.

Discussion

We suggest that most vesicles do not participate directly in neurotransmitter release and vesicle recycling. The number of vesicles functioning in vitro has been much discussed in the last decades, with many conflicting studies. Because of space limitations we cannot delve into the large literature on the subject [see reviews (2, 32)]. Generally, NMJ works conclude that many or most vesicles can be used under stimulation, although some vesicle pools are used preferentially (see, for example, the work of Kuromi and Kidokoro on the *Drosophila* NMJ, or the work of Betz and collaborators on the frog NMJ; reviewed in ref. 2), whereas the remaining vesicles constitute a reserve pool. Tsien and colleagues (3) concluded that a substantial proportion of the vesicles in hippocampal neurons remain unused even during strong nonphysiological stimulation (reviewed in ref. 2), albeit other investigations suggested that all vesicles may be releasable (6).

The classic reserve pool of the literature typically comprises 50–80% of all vesicles. This concept can hardly apply to an in vivo situation in which we find more than 90% to be "in reserve" or else to serve another purpose. It is difficult to imagine physiological situations that would require synapses to release substantially more vesicles at any one time. For example, the ventral muscles of the *Drosophila* larva would certainly be used more frequently if the larva attempted to avoid a predator, but with *Drosophila* movement being inherently slow, no great rates of activity could ever be attained. Similarly, stress increased the use of vesicles in locusts (Fig. 3), but even in this life-or-death situation the jumping behavior was infrequent. Importantly, because predation eventually resulted in the death of all locusts analyzed, no higher stimulation could take place in vivo—no life situation could have induced a stronger need for movement (and hence vesicle recycling). Finally, as indicated in the Introduction,

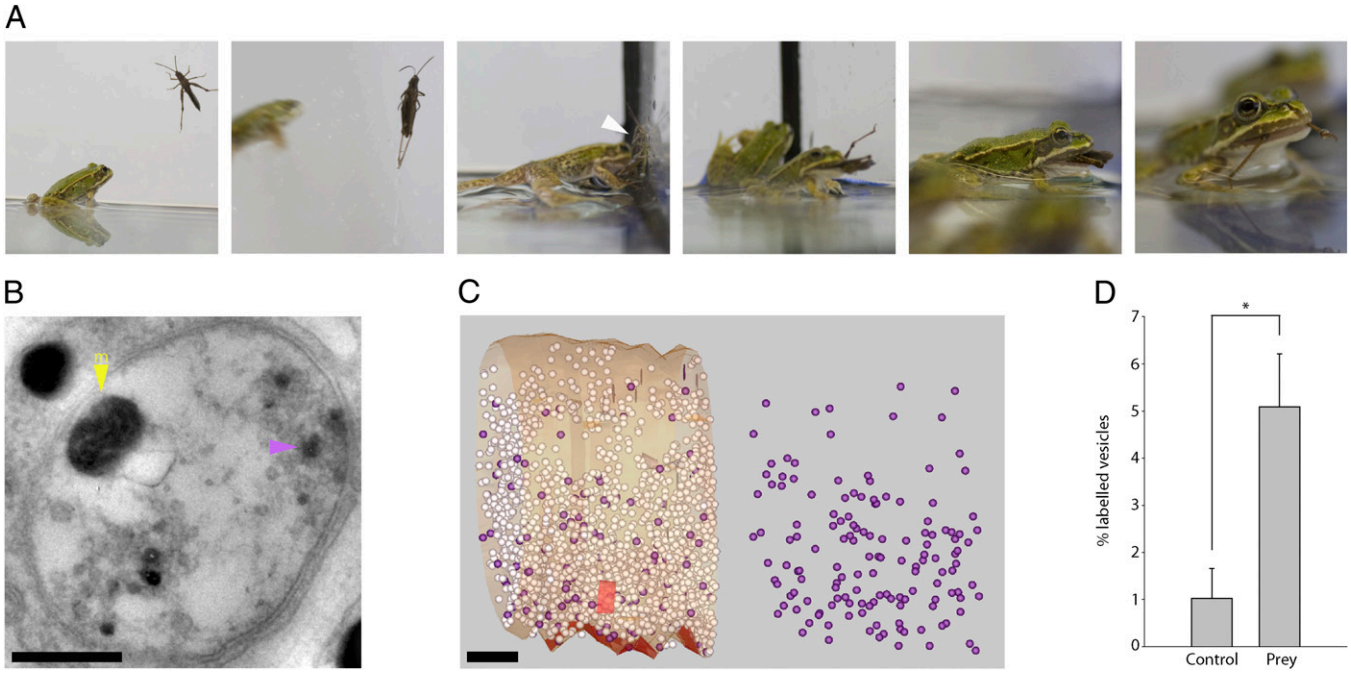

Fig. 3. Limited use of vesicles under extreme physiological stimulation. (*A*) Representative images of predator/prey experiment. One injected locust was placed into a terrarium with several frogs. The arrowhead in the third panel (from left) points to the position of the locust immediately after a failed capture attempt (the head of the frog is behind the locust). The locust was caught and eventually ingested after 5–10 min (*Right*). (*B* and *C*) Electron micrograph and 3D reconstruction of a nerve terminal from a hunted locust. (Scale bars, 400 nm in *B*, 300 nm in *C*.) (*D*) Percentage of labeled vesicles (four to eight independent preparations, means ± SEM; $P < 0.05$, *t* test).

the rates of activity cannot increase endlessly/continually, at least for the NMJs, because the muscles would stop responding.

The reserve vesicles seem to be clustered and are therefore rather immobile (Fig. 4). They would simply stay linked to other vesicles and/or to the cytoskeleton (perhaps via synapsin), at tens or hundreds of nanometers from the active zones. This hypothesis is in agreement with two recent findings on vesicle mobility and recycling: first, only a small pool of vesicles is mobile in cultured hippocampal synapses, with all other vesicles immobile ["fixed" (33)]. The fixed vesicles did not become more mobile upon physiological stimulation, suggesting that they may not be involved in neurotransmitter release and recycling under normal circumstances (see also ref. 30). Second, in the same preparation only few active (mobile) vesicles have their membranes optimally sorted via endosomes (34). All other vesicles were found to be reluctant to release and did not seem to use endosomes when forced to release via prolonged stimulation in vitro. It is tempting to hypothesize that the reason why most synaptic vesicles do not sort their membranes optimally (via endosomes) is that these vesicles are not normally intended for recycling (in vivo).

Our data do not exclude the existence of synapses that use most of their vesicles. They also clearly indicate that the pool tags are not permanent, with the active and inactive vesicles intermixing slowly over time (Fig. S1), so that in a strict sense all vesicles may be eventually used in neurotransmitter release. Additionally, the intermixing might be faster at higher temper-

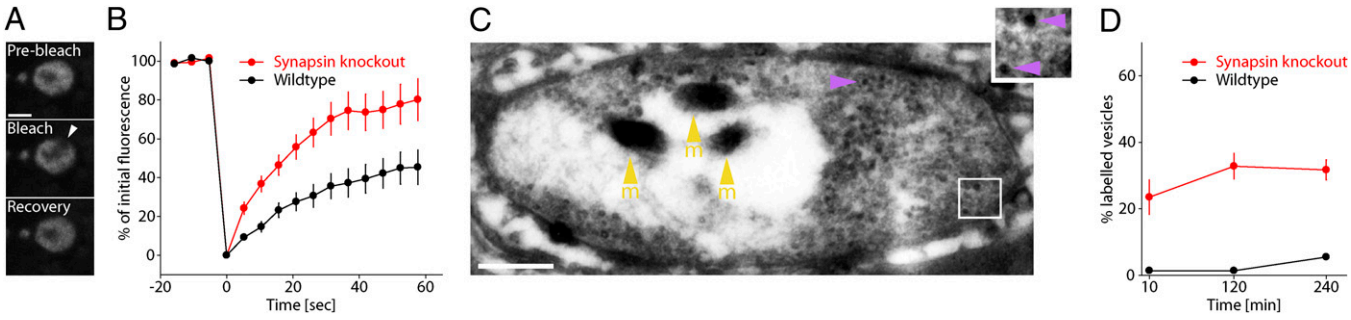

Fig. 4. Vesicle mobility and the number of actively recycling vesicles are higher in synapsin-knockout *Drosophila* larvae. (*A*) FRAP experiments in *Drosophila* larval NMJs. Third instar larvae (wild-type or synapsin knockout) were dissected and stimulated in the presence of 10 μM FM 1-43 (30 Hz/10 s). Afterward a small region was bleached within the bouton (arrowhead), and the recovery of fluorescence was measured over 1 min. (Scale bar, 2 μm.) (*B*) Quantification of fluorescence recovery. Means of 35 (wild-type) and 49 (synapsin knockout) recovery curves are shown (±SEM), from six independent experiments each. The fluorescence recovery is significantly higher in the synapsin-knockout boutons ($P < 0.005$, Kolmogorov-Smirnov test). (*C*) Electron micrograph of a synapsin knockout larval terminal 4 h after FM 1-43 injection. Yellow arrowheads indicate three mitochondria; purple arrowheads point to labeled vesicles. (Scale bar, 400 nm.) (*D*) Percentage of labeled vesicles at different time points after injection in synapsin-knockout larvae. The wild-type time course is the same as in Fig. S1*A*. Note that the error bars are occasionally smaller than the symbol size.

atures (at least for invertebrates, which were maintained at 21 °C in our experiments). However, at any point in time only a small proportion of the vesicles recycle. It is therefore unlikely that physiological activity would deplete all vesicles at one time, which is the only event that would justify the need for a large reserve pool of vesicles. In a related study published in this issue (35), we present the hypothesis that these vesicles support release indirectly, functioning primarily as a molecular buffer for proteins involved in vesicle recycling. Thus, all vesicles seem to have some role in synaptic physiology, although only a minority is actually involved in neurotransmitter release at any one time.

Methods

Materials, animals, reagents, and previously published methods are described in detail in *SI Methods*. Dye injection in living animals was performed to obtain a final concentration of approximately 10 μM FM 1-43 in the animal, using either a FemtoJet express microinjecting device (Eppendorf) for the small animals (*C. elegans*, *Drosophila* larvae) or 1-mL syringes (Terumo) equipped with 0.3 × 20-mm needles (catalog no. 13.201-09, Unimed) for all other animals. The injection was performed in the body cavity (invertebrates) or s.c. (vertebrates). See *SI Methods* for further details. During the time between injection and dissection, animals were generally kept in an environment that allowed them to move, eat, and sleep. Dissections were then performed in ice-cold Ca^{2+}-free buffer according to standard procedures (detailed in *SI Methods*). After dissection, preparations were fixed for 30 min in 2.5% glutaraldehyde (in PBS) on ice, followed by further fixation for 30 min to 1 h in 2.5% glutaraldehyde at room temperature. The subsequent photooxidation steps were performed as previously described (20). Electron microscopy procedures were performed as previously described (20). PHluorin imaging was performed largely as previously described (23). The comparison of synaptic vesicle release and recycling was performed by monitoring release electrophysiologically (as described in ref. 37) in the presence of FM 1-43. After a period of ≈10 min the preparations were fixed, photooxidized, and processed for electron microscopy, as above; see *SI Methods* for more details.

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