ISSN 0006-2979, Biochemistry (Moscow), 2026, Vol. 91, No. 4, pp. 561-587 © Pleiades Publishing, Ltd., 2026.

REVIEW

Functioning of Synaptic Vesicle Pools: Diversity and Organizational Principles

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Received February 25, 2026
Revised March 26, 2026
Accepted March 26, 2026

Abstract—Presynaptic nerve terminals contain a large number of vesicles filled with neurotransmitters, whose release ensures signal transmission from the presynaptic neuron to the postsynaptic cell. Despite their morphological homogeneity, synaptic vesicles (SVs) are functionally heterogeneous and are organized into distinct groups (pools) that differ in their ability for exocytosis and mobilization, recycling kinetics, and protein composition. In addition to the classic pools – the readily releasable pool (RRP), recycling pool, and reserve pool – other populations have been identified, including spontaneously recycling vesicles, vesicles of resting pool and superpool. Vesicles from different pools engage in different modes of exocytosis and endocytosis, and the extent of interpool mixing varies depending on the synapse type and physiological or pathological conditions. Changes in the organization of SV pools underlie multiple forms of synaptic plasticity. Furthermore, SV cycling is a target of several pharmacological agents, and its disruption plays a significant role in the pathogenesis of neurodegenerative diseases. This article is a systematic review of SV pools, their organizational features in central and peripheral synapses, and implications of changes in the structure of SV pools in synaptic plasticity, action of drugs, and development of neurological disorders.

DOI: 10.1134/S0006297926600535

Keywords: active zone, vesicle recycling, synaptic vesicle, presynaptic nerve terminal, synaptic vesicle pool, exocytosis, endocytosis

INTRODUCTION

In the nervous system, signal transmission from a presynaptic cell to a postsynaptic cell occurs primarily at chemical synapses, where the two cells are separated by a synaptic cleft. This process typically takes a fraction of millisecond to several milliseconds and proceeds via release of neurotransmitters from the presynaptic terminal and their subsequent binding to receptors on the postsynaptic membrane. In the presynaptic terminal, neurotransmitter molecules are stored in 40 to 50-nm synaptic vesicles (SVs).

SV cycling is a fundamental mechanism underlying neurotransmission. In response to the action potential, vesicles undergo exocytosis, resulting in rapid (less than 1 ms) release of neurotransmitters into the synaptic cleft. Vesicle endocytosis and reloading with neurotransmitter molecules occur within tens of seconds or even minutes and replenish the pool of SVs available for subsequent rounds of exocytosis [1].

Despite being morphologically similar, SVs are functionally and biochemically heterogeneous and can be classified into distinct populations (pools). The general vesicle pools include the readily releasable pool (RRP), which is the first to undergo exocytosis; the recycling pool, which sustains neurotransmission during moderate activity; and the reserve pool, which is mobilized during periods of intense synaptic activity [2]. Recent studies have identified more specialized vesicle populations, including spontaneously recycling vesicles [3], the resting pool, which does not normally participate in neurotransmitter release [4],

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and the "superpool" containing the vesicles capable of exchanging between synaptic boutons [5].

Some pools can overlap; for example, vesicles from the reserve pool may transit to the superpool and vice versa [5]. Other pools are less prone to intermix and retain their identity even after multiple rounds of exo- and endocytosis. SVs from different pools exhibit functional heterogeneity, as they can release neurotransmitter and then be recovered via different modes of exocytosis (synchronous, asynchronous, spontaneous) and endocytosis (clathrin-dependent, kiss-and-run, ultrafast, and bulk), respectively. Vesicles from different pools may also differ in molecular composition, which likely contributes to the functional specialization of vesicle populations [6].

The organization of vesicle pools varies across synapses, reflecting adaptations to specific patterns of synaptic activity. Changes in the structure of SV pools underlie synaptic plasticity. Moreover, SV cycling is a target for various pharmacological agents, such as fluoxetine [7] and atorvastatin [8]. Disruptions in the vesicle cycling are implicated in the pathogenesis of neurological disorders, such as Alzheimer's disease [9], Parkinson's disease [10, 11], schizophrenia [12], and amyotrophic lateral sclerosis (ALS) [13, 14].

This article discusses the known SV pools, their properties and functional features, and the modes of exo- and endocytosis associated with each pool, as well as examines the features of SV pool organization in various systems, such as hippocampal synapses, calyx of Held synapses, *Drosophila*, frog and mouse neuromuscular junctions (NMJs), and ribbon synapses. We also addressed changes in the organization of SV pools during the induction of synaptic plasticity, under the action of biologically active substances, and in neurological disorders.

READILY RELEASABLE POOL (RRP) OF SYNAPTIC VESICLES

The RRP comprises a small fraction of SVs (typically 1-2% of the total). Vesicles of this pool are the first to undergo exocytosis upon the presynaptic terminal activation (Fig. 1). Most RRP vesicles are docked at the presynaptic membrane in a specialized region adapted for exocytosis, known as the active zone (AZ) [2, 15-18]. SNARE proteins in these vesicles and in the AZ are partially coiled and are in a fusion-ready (primed) state [19]. However, docking does not necessarily imply priming: not all docked vesicles belong to the RRP. For example, in cultured hippocampal neurons, the number of RRP vesicles is lower than the number of docked vesicles [15]. Conversely, not all RRP vesicles are docked. Thus, frog NMJs contain a subset of RRP vesicles in the cytoplasm of the nerve terminal [20], suggesting the existence of an ultrafast mechanism that rapidly recruits these SVs to sites of exocytosis.

In the central nervous system (CNS), stimulation of a single synapse often fails to trigger the neurotransmitter release. In this case, the probability of evoked vesicle exocytosis at the nerve terminal depends on both the RRP size and the release probability of an individual vesicle within RRP [21]. Therefore, modulation of the RRP size plays an important role in the formation of synaptic plasticity and regulation of synaptic strength.

SVs in the RRP are not functionally uniform; rather, the pool exhibits heterogeneity (Fig. 1). Studies conducted in mouse NMJs have shown that during high-frequency stimulation, the number of RRP vesicles is roughly equivalent to the number of docked SVs. In contrast, during low-frequency stimulation, the size of the RRP is substantially smaller and represents only a fraction of the docked vesicle population. This suggests that in mouse NMJs, some docked vesicles may remain functionally silent during low-frequency stimulation, but are released as a part of the RRP during high-frequency stimulation, ensuring more efficient synaptic transmission under these conditions. One possible explanation for this fact is that not all AZs are engaged during the low-frequency stimulation. Certain AZs may form clusters (multimeric AZs) characterized by a high density of Ca^{2+} channels and, consequently, a higher probability of SV release. Under low-frequency stimulation, neurotransmitter release may occur from these multimeric AZs, while unitary AZs remain dormant and are recruited only during high-frequency stimulation [22]. Alternatively, heterogeneity may arise from differences in the vesicle molecular composition and intrinsic Ca^{2+} sensitivity [23]. Consistent with this view, maturation is associated with an increase in the RRP size in mouse NMJs, accompanied by a more diffuse distribution of SVs within the axoplasm [24].

In calyx of Held synapses, neurotransmitter release from the RRP exhibits two components – fast and slow – reflecting the presence of two vesicle subpopulations with different sensitivity to cytosolic Ca^{2+} [25]. This heterogeneity contributes to the short-term plasticity: the fraction of rapidly releasable vesicles increases after tetanic stimulation, thereby increasing the number of released vesicles at the onset of subsequent stimulation [26].

A similar vesicle heterogeneity is also observed in synapses formed between granule cells and Purkinje cells in the cerebellum. The presynaptic terminals of granule cells contain two SV subpopulations in the RRP. One of them is released in response to single stimuli, but ceases to be released during the following low-frequency stimulation, likely

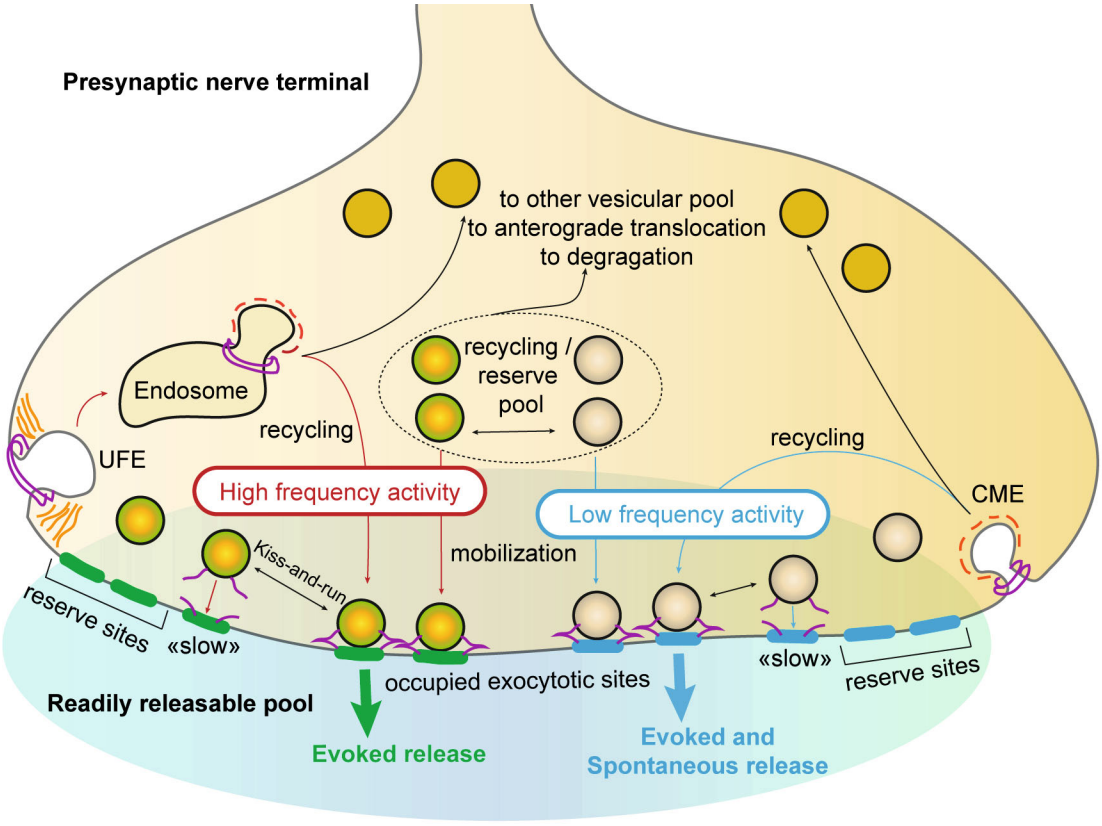

Fig. 1. The functioning of SVs of the RRP depends on its organization and replenishment mechanisms. Most RRP vesicles are primed in the AZ, while a smaller fraction resides away from the membrane but can rapidly dock at exocytosis sites in response to neuronal activation. Certain regions of the presynaptic terminal may also serve as precursors for the formation of new exocytosis sites during synaptic plasticity induction. Some RRP vesicles release neurotransmitter in response to low-frequency stimulation and may also undergo spontaneous fusion, contributing to spontaneous exocytosis. Other RRP vesicles are preferentially engaged during high-frequency stimulation, reflecting differences in both vesicle properties and characteristics of associated exocytosis sites. RRP replenishment can occur through the mobilization of vesicles from other pools (e.g., recycling and reserve pools) and recycling by endocytosis. The prevalent endocytosis mechanism for RRP replenishment can vary across different synapse types. Clathrin-mediated endocytosis (CME), which is a relatively slow process, primarily sustains vesicle supply during low-frequency activation, while ultrafast endocytosis (UFE) followed by endosomal sorting enables rapid recovery of vesicles after brief bursts of high-frequency stimulation. Additional mechanisms may also contribute to vesicle recycling, such as kiss-and-run exocytosis, in which vesicles transiently fuse and rapidly detach without full collapse into the presynaptic membrane. Following endocytosis, vesicles can be redistributed to another pool, targeted for degradation, or transported to the soma for signaling functions. Dynamic exchange between vesicle pools also takes place.

acting as a filter for weak or "insignificant" stimuli. The second subpopulation is recruited only during the high-frequency stimulation and remains largely inactive at low frequencies. This RRP fraction is characterized by faster recycling, ensuring more efficient neurotransmission during high-frequency activity [27, 28].

The mechanisms underlying RRP heterogeneity remain poorly understood. Contributing factors likely include the number of voltage-gated Ca^{2+} channels at individual exocytosis sites and their properties (post-translational modifications, interaction with partner proteins, subunit composition), location of docked synaptic vesicles relative to Ca^{2+} channels [29-31], different extent of vesicle priming in the AZ (e.g., superpriming [32]), the properties of exocytosis sites themselves, protein and lipid composition of SVs [33], and local conditions, such as nearby spontaneous exocytosis events, random opening of Ca^{2+} or K^+ channels, etc. The factors determining SV heterogeneity likely vary in different synapses, have their own properties, and dynamically adjust in response to ongoing patterns of synaptic activity.

Changes in the RRP structure. Changes in the number of SVs, as well in the proportion of active SVs in the RRP, play an important role in synaptic plasticity. For example, in hippocampal neurons, activation of cAMP-dependent protein kinase A pathway, which induces long-term potentiation (LTP), leads to the increase in the number of docked SVs

in the AZ [34] together with remodeling of the AZ ultrastructure, when docked "non-releasable" SVs from the reserve pool are replaced with active RRP vesicles [35]. High-frequency stimulation, as well as activation of the Sema3a–PlexinA4–ITGB1 pathway involved in presynaptic plasticity, cause an increase in the number of RRP vesicles by mobilizing SVs from the reserve pool [35, 36]. Expansion of the RRP after LTP induction increases the number of SVs available for release, thereby improving the reliability of synaptic transmission, and is necessary for the formation of stronger connections [35]. Activation of $GABA_B$ receptors in the nerve terminals of the medial habenula causes a 4-fold increase in the RRP size and the number of docked vesicles due to the translocation of CAPS2 proteins (Ca^{2+}-dependent activator protein for secretion 2) associated with additional vesicles to the AZ within a few minutes [37]. Ca^{2+}-dependent accumulation of SVs in the RRP has been implicated in the short-term facilitation of neurotransmitter release during paired stimulation in *Drosophila* NMJs [38].

The opposite scenario is also possible: vesicles can leave the RRP and localize within 100 nm of the AZ. These SVs can reoccupy free exocytosis sites, although they may alternatively relocate to the reserve pool [39]. Some SVs can undergo rapid (within 100 ms) undocking from the AZ. Such undocking events are several times more frequent than spontaneous exocytosis [40]. In hippocampal synapses, stimulation can trigger the undocking of certain vesicles from the presynaptic membrane instead of fusion [41]. In some types of central synapses, repeated low-frequency stimulation leads to synaptic depression. One possible mechanism underlying this effect is reduction in the RRP size driven by vesicle undocking and transition to another pool [42].

Disruptions in the RRP structure are observed in certain pathologies. For example, in epilepsy, inhibitory synapses show an increased number of docked SVs, while the proportion of fusion-ready (primed) SVs among them decreases [43], indicating a reduction in the RRP size.

RRP replenishment. The RRP can be replenished through multiple pathways, including recruitment from reserve and recycling pools, as well as endocytosis (Fig. 1). In large synapses, such as calyx of Held synapses, RRP replenishment occurs via vesicle recruitment from the recycling or reserve pool, providing their continuous supply to the AZ [44-46]. Synapses between parallel fibers and interneurons of the cerebellar molecular layer contain the so-called intermediate, or upstream, pool. Positioned functionally between the recycling pool and the RRP (i.e., upstream of the RRP), this pool contains a small number of SVs (1-4 vesicles per release site or 6-20 vesicles per AZ) and serves as a source for RRP replenishment. It is quickly depleted (within ~10 action potentials) and is restored within 0.5-1 s, likely via vesicle recruitment from the recycling pool. Such replenishment dynamics is essential for maintaining reliable responses to temporally separated bursts of stimuli [47]. Furthermore, the intermediate pool is recovered through a fast, dynamin-dependent endocytotic pathway that operates on a timescale of ~10 s and can generate up to ~200 vesicles per AZ, thus preventing excessive depletion of the recycling pool [48].

In hippocampal synapses, the RRP can be maintain by recycling for a relatively long time without SV mobilization from the reserve pool, i.e., RRP replenishment occurs through endocytosis [49]. One possible explanation is that these synapses contain a relatively small number of vesicles, so the efficiency of the existing endocytic machinery is sufficient for rapid and efficient SV recycling. Furthermore, the mechanism of endocytosis varies between the synapse types. In inhibitory hippocampal synapses, the kiss-and-run endocytosis predominates, whereas excitatory synapses primarily rely on clathrin-mediated endocytosis [50]. Interestingly, in postganglionic sympathetic nerve terminals, exocytosis of RRP vesicles is coupled with a mechanism ensuring rapid reuptake of catecholamines into the presynaptic terminal for RRP vesicle refilling [51].

The mechanisms by which endocytosis replenishes the RRP vary depending on the synapse type and physiological conditions, including patterns of neuronal activity. In presynaptic terminals, the predominant pathway is clathrin-mediated endocytosis. Following vesicle fusion with the presynaptic membrane, vesicular components are redistributed to endocytosis sites (peri-AZs), where vesicles bud off via a clathrin-dependent process. In cortical and hippocampal synapses, as well as in cultured neurons, released vesicles can be retrieved via clathrin-mediated endocytosis, thus replenishing the RRP [52, 53]. Clathrin-mediated endocytosis operates efficiently during moderate levels of synaptic activity. In contrast, at higher stimulation frequencies, faster endocytotic mechanisms, such as kiss-and-run and ultrafast endocytosis, predominate, enabling more rapid recycling of RRP vesicles [54-56].

In the kiss-and-run mechanism, neurotransmitter molecules are released from the vesicles through a small protein pore, so vesicles detach from the membrane without fusing with it [57]. Vesicle detachment occurs with the participation of the GTPase dynamin and similar mechanoenzymes, whose oligomers form a constricting "collar" at the neck between the SV and AZ membrane. In cerebrocortical terminals and hippocampal neurons, these rapid cycles of exocytosis and endocytosis (kiss-and-run) can sustain RRP recycling for a long time [58-60].

Ultrafast endocytosis also ensures rapid SV recycling, but operates via a different mechanism. In this case, vesicles fully fuse with the membrane, after which the membrane is retrieved through the actin-dependent formation of endocytotic invaginations that are routed into endosomes, from which new vesicles subsequently bud off [16, 61]. This process is highly temperature-sensitive ($Q_{10} \approx 3.5$) and is observed primarily at physiological temperatures [62].

In addition to fast endocytosis pathways, there is a mechanism that accelerates vesicle reformation from fragments of the presynaptic membrane. The presynaptic membrane contains specialized microdomains with pre-sorted and clustered SV proteins, referred to as readily retrievable pool (RRetP) [63]. These regions contain vesicular proteins, such as synaptotagmin 1, synaptobrevin 2, and synaptophysin 1, and are also associated with clathrin and adaptor proteins, such as AP180 and stonins [64]. It is likely retention of vesicular proteins in the limited regions of the presynaptic membrane is provided by clathrin and adaptor proteins, along with the lipid raft-dependent aggregation and interaction with cytoskeletal elements. Such presorting ensures a high rate of clathrin-dependent endocytosis during the initial cycles of SV exo- and endocytosis. Thus, the functioning of RRetP can explain that in frog NMJs, the rate of clathrin-dependent endocytosis during the first 10 s of activity is significantly higher than its typically observed rate [63, 65].

TYPES OF NEUROTRANSMITTER RELEASE AND THE RRP

The types of neurotransmitter release include the following: evoked synchronous, evoked asynchronous, and spontaneous. RRP vesicles mediate synchronous neurotransmitter release because they are already docked at the membrane and primed for fusion. Consequently, the influx of Ca^{2+} through voltage-gated calcium channels in the AZ can trigger their fusion extremely rapidly (in less than 1 ms) following the presynaptic spike. It was shown that in the hippocampus, synchronous vesicle fusion causes a significant decrease in the number of SVs docked to the AZ membrane [41]. Mutations leading to impaired vesicle docking also reduce synchronous vesicle fusion [66-68]. Additionally, evidence indicates that in hippocampal neurons, the RRP is preferentially replenished by synchronously cycling vesicles [69].

The RRP is also involved in spontaneous neurotransmitter release, which may be caused by stochastic fusion of SVs already in a fusion-competent state [70]. In support of this hypothesis, studies in the hippocampus have revealed that an increase in the RRP size is associated with a with a higher frequency of spontaneous neurotransmitter release [71-74], while a decrease in the RRP size leads to a corresponding decrease in the number of spontaneous release events [75]. Moreover, reduced stability of docked SVs, such as that induced by mutations in synaptotagmin 1 (the main Ca^{2+} sensor in exocytosis), promotes spontaneous vesicle fusion [76, 77].

However, it remains unclear whether the same pool mediates both spontaneous and evoked release, or there exists a distinct pool that provides most spontaneous exocytosis events without contributing to the evoked neurotransmitter release [78, 79]. Current evidence supports both possibilities. One potential explanation is the existence of two vesicle populations, one of which is released constitutively regardless of stimulation (constitutively releasing vesicles, or CRVs) and the other is primarily responsive to stimulation, although vesicles of this population can also fuse spontaneously, i.e., acting as spontaneously releasing synaptic vesicles (SRSVs) [80]. It is possible that some RRP vesicles may belong to the SRSV pool.

The asynchronous release is mainly provided by the recycling and reserve pools. However, in calyx of Held synapses, some RRP vesicles undergo asynchronous release during prolonged stimulation [81]. Possible molecular mechanisms of asynchronous neurotransmitter release and its temporal dynamics are described in detail in reviews [82, 83].

SYNAPTIC VESICLE POPULATIONS UNRELATED TO THE RRP

In the first approximation, the reserve pool can be defined as all SVs not included in the RRP. However, within this broad definition, several functionally distinct subpools can be identified based on their release properties and role in synaptic transmissions (Fig. 2).

(I) **The recycling pool** sustains synaptic transmission after RRP depletion. Vesicles of the recycling pool can be rapidly mobilized to the AZ and undergo recycling during moderate-frequency activity, supporting neurotransmission for a long time without involving other SV populations.

(II) **The reserve pool proper** supplies vesicles to the recycling pool when the latter becomes depleted and is mainly engaged during intense, prolonged activity.

(III) **The spontaneously recycling pool** includes vesicles that spontaneously release neurotransmitter, independent of the action potential.

(IV) **The resting pool** contains vesicles that do not release neurotransmitter under any condition and likely serves as a reservoir of proteins and lipids to meet the local needs of the presynaptic apparatus.

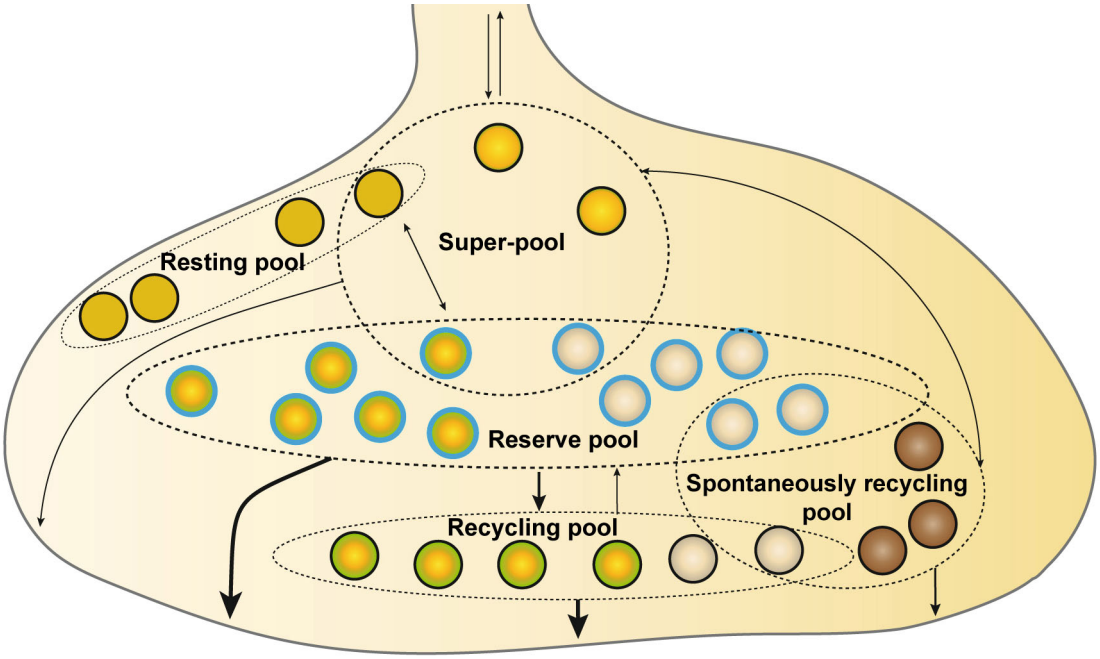

Fig. 2. SV pools in the cytoplasm of the nerve terminal (excluding the RRP). The recycling pool sustains neurotransmission during most synaptic activity regimes *in vivo*. However, during high-frequency stimulation, prolonged moderate activity, or when vesicle recycling is impaired, vesicles from the reserve pool (majority of SVs in the terminal) are recruited to support neurotransmission. Vesicles of the resting pool do not participate in recycling under normal conditions and serve as a reservoir for proteins, lipids, and small molecules in the nerve terminal. Vesicles of the superpool are characterized by a high mobility and can traffic between the AZs and synaptic contacts along the axon, enabling relatively rapid redistribution of vesicles between distant exocytosis and endocytosis sites. Vesicles involved in evoked exocytosis, together with a distinct population that is largely unresponsive to stimulation, may contribute to a pool that predominantly mediates spontaneous neurotransmitter release.

(V) **The superpool** (~4% of the total vesicle population in a synapse) [5] comprises vesicles that traffic between synaptic boutons, ensuring the optimal distribution of SVs between different, even distant, AZs.

The classification of SVs into distinct pools is somewhat conditional. First, the same SVs can belong to functionally different pools, i.e., mixing between pools is possible. For example, vesicles from the recycling and reserve pools can be released not only in response to stimulation but also spontaneously, thereby contributing to multiple functional pools. Second, vesicles can transit between pools during synaptic activity. During prolonged, high-frequency stimulation, vesicles from the reserve pool are recruited into the recycling pool. Both pools can supply vesicles to the superpool, while reverse transition is also possible. In addition, vesicle precursors are continuously delivered to the nerve terminal, where they mature into functional SVs after several rounds of exocytosis and endocytosis. At the same time, older vesicles are transported back to the soma for degradation. This ongoing turnover ensures continuous renewal and alters the relative sizes of SV pools. Overall, intense synaptic activity promotes mixing between vesicle pools and facilitates adaptive changes in their relative proportions to meet the demands of synaptic transmission.

THE RECYCLING POOL

The recycling pool includes a relatively small number of SVs (less than 20% of the total) distributed throughout the presynaptic terminal [2, 84].

The mechanisms of recycling pool replenishment vary across the synapse types and activity modes. In frog NMJs, the recycling pool sustains neurotransmission during the low-frequency stimulation (2-10 Hz) and brief periods of high-frequency activity (tens of seconds at 20-30 Hz), primarily through the reuse of endocytosed vesicles. However, during prolonged high-frequency stimulation, the recycling pool becomes depleted and is replaced by vesicles from the reserve pool, which are recruited more slowly into exocytosis, leading to a reduction in the quantal content [85, 86]. In native hippocampal synapses, the same vesicles support neurotransmission over extended periods of time by forming a relatively closed recycling pool maintained by endocytosis [87]. In contrast, in calyx of Held synapses, reserve pool vesicles

contribute to the recycling pool replenishment even during the low-frequency activity [45, 46].

Rapid replenishment of the RRP by the recycling pool may depend on the interactions between SVs and proteins intersectin 1 and endophilin. In hippocampal synapses, these proteins form a dynamic condensate that associates with SVs in the region between the RRP and reserve pool (often referred to as the replacement zone) and promote the positioning of vesicles in close proximity (within ~20 nm) to the AZ. In the absence of intersectin 1, fewer SVs are found near the AZ, and vacant release sites are filled more slowly, leading to the inhibition of neurotransmitter release [88].

Vesicles of the recycling pool participate in synchronous, asynchronous, and spontaneous neurotransmitter release. In the hippocampus, the recycling pool can sustain synchronous neurotransmitter release over extended periods of time. However, during high-frequency stimulation, when not all release sites can be filled with docked vesicles, neurotransmission is maintained through the asynchronous release of vesicles from both the recycling and reserve pools [89]. Additionally, several studies indicate that vesicles from the recycling pool also participate in spontaneous neurotransmitter release [90, 91].

THE RESERVE POOL

Vesicles of the reserve pool are distributed throughout the presynaptic terminal and are generally not spatially segregated from those of the recycling pool [79, 92, 93]. The ratio of the reserve and recycling pools can vary among different synaptic boutons in the CNS [94]. Unlike the recycling pool, reserve pool vesicles are mobilized only during prolonged or high-frequency stimulation, thus, suggesting the presence of mechanisms ensuring their functional differentiation from the recycling pool. One such mechanism involves association with synapsins [95], proteins regulating vesicle mobility [96]. Interaction with these proteins is a distinctive feature of the reserve pool, as evidenced by studies showing that the synapsin gene knockout leads to a marked deficiency of reserve pool vesicles [97-99] and results in rapid synaptic depression during repeated high-frequency stimulation [100]. Synapsins bind reversibly to SVs and to each other, promoting vesicle clustering. Vesicle retention may occur through two main mechanisms: (i) formation of tetrameric cross-links mediated by synapsin I, and/or (ii) multivalent interactions among synapsin molecules that drive phase separation in the cytosol, forming a “synapsin phase” in which SVs are embedded. The first mechanism appears to be more efficient and may dominate in excitatory synapses, whereas the second one is more likely to occur in inhibitory synapses [101].

SVs associated with synapsin are released only when there is a sufficiently large increase in cytosolic Ca^{2+}, which typically occurs during intense activity. One possible explanation for why the reserve pool is not recruited at low and medium stimulation frequencies is that it includes vesicles containing proteins damaged during their previous recycling, as observed in hippocampal neurons [102].

Most reserve pool vesicles are not docked at the membrane and, therefore, require additional time to be transported to the AZ. This delayed recruitment contributes to their asynchronous release in hippocampal synapses [103], where reduced asynchronous release has been linked to the accumulation of vesicles in the reserve pool. Furthermore, reserve pool vesicles exhibit heterogeneity in their mobilization rates: “fast” and “slow” mobilizable vesicles replenish different subpopulations of the RRP, supporting fast and slow components of neurotransmitter release (the parallel model of vesicle delivery [104]). In mouse NMJs, the reserve pool contributes not only to asynchronous release but also to synchronous exocytosis [99, 105].

Vesicles of the reserve pool may vary slightly in protein composition from those in the recycling pool, indicating biochemical heterogeneity among SVs. These differences can explain, at least partially, the existence of different (spontaneous or evoked) release mechanism. For example, reserve pool vesicles in the hippocampal neurons contain relatively high amounts of the SNARE protein VAMP7, which is associated with spontaneous exocytosis, whereas VAMP2, a SNARE protein involved in evoked exocytosis, is present at lower levels. Consequently, although these vesicles are capable of stimulus-induced release, their enrichment with VAMP7 “biases” them toward spontaneous neurotransmitter release [91, 106]. Interestingly, vesicles generated after asynchronous release induced by high-frequency stimulation are more prone to Ca^{2+}-sensitive spontaneous release, because both processes involve the same SNARE protein, VAMP4 [107].

The reserve pool is replenished through bulk endocytosis (also known as activity-dependent bulk endocytosis, ADBE), as demonstrated in many synaptic models, including CNS neurons [52], calyx of Held synapses [84], and *Drosophila* and frog NMJs [108]. It involves membrane invagination followed by the pinching off of cisternae, from which SVs subsequently bud off and are delivered to the reserve pool. It is believed that this bulk endocytosis is necessary to preserve the presynaptic membrane structure during periods of intense stimulation of exocytosis by maintaining its biochemical composition and tension.

However, this pathway can also permit the entry of high-molecular-weight structures, such as toxins and viruses, into nerve terminals [109, 110].

Bulk endocytosis is activated in the same way as mobilization of reserve pool SVs during high-frequency stimulation [111]. Evidence suggests that these processes share regulatory mechanisms [112]: during prolonged or intense stimulation, Ca^{2+} levels in the neuron terminal rise, leading to the activation of calmodulin and, hence, calcineurin (protein phosphatase 2B) stimulation. Calcineurin dephosphorylates synapsin, followed by its rephosphorylation by MAP kinase and/or Cdk5, resulting in the modulation of the reserve pool vesicle release. Concurrently, calcineurin dephosphorylates dynamin 1, a key step in initiating bulk endocytosis. However, bulk endocytosis is not the sole pathway for replenishing the reserve pool. In calyx of Held synapses, all endocytosed vesicles replenish the reserve pool, regardless of the type of endocytosis [46].

THE SUPERPOOL AND THE RESTING POOL

The superpool is partially "extrasynaptic," as it represents a population of vesicles transported along the axon between neighboring synaptic terminals in an actin-dependent manner [114]. The superpool is formed from vesicles of the reserve and recycling pools, which retain their ability to release neurotransmitters after relocation to other areas [5]. The mobility of SVs is limited due to their binding to synapsin: disruption of synapsin functional activity leads to a massive diffusion of SVs from synaptic boutons into axons, significantly increasing the size of the superpool [115]. Another regulator of SV mobility is the vesicular glutamate transporter (VGLUT1), whose main function is to load neurotransmitter molecules into SVs. VGLUT1 also promotes retention of SVs within the presynaptic terminal, but the underlying mechanisms remain obscure. Presumably, increased SV loading, mediated by VGLUT1, limits their mobility. Alternatively, VGLUT1 may retain SVs in the synapse through protein–protein interactions, for example with endophilin [116]. The superpool likely serves as a reservoir for redistributing neurotransmitter-containing vesicles between active and inactive synapses, thereby contributing to synaptic plasticity. An increase in the number of SVs in active synapses is associated with potentiation, whereas depletion at inactive synapses contributes to synaptic depression [114].

Some synapses contain a distinct pool of vesicles that remains non-releasable under any conditions. Such pool constitutes a substantial fraction of vesicles contained in presynaptic terminals. In calyx of Held synapses, these vesicles account for ~20% of the total pool [117], whereas in hippocampal neurons, this proportion may reach up to 90%, according to some estimates [118]. The functional role of this pool remains debated. Studies in mouse NMJs indicate that it may serve as a reservoir of proteins required for SV recycling. During periods of intense stimulation, vesicles from this pool are not released; instead, associated soluble proteins are mobilized [119]. However, it is still unclear whether the resting pool can be fully mobilized. Other studies have reported that in both calyx of Held synapses and the hippocampus, complete depletion of all vesicles can occur even during prolonged low-frequency stimulation [4, 120].

SPONTANEOUS NEUROTRANSMITTER RELEASE AND SYNAPTIC VESICLE POOLS

Several studies suggest that spontaneous neurotransmitter release is mediated by a distinct pool of spontaneously recycling vesicles. For example, experiments in inhibitory hippocampal neurons identified two vesicular pools: one released in response to stimulation, and the other released predominantly spontaneously [3]. Conversely, other studies indicate that a single vesicle pool may provide both spontaneous and evoked neurotransmitter release [121]. However, even in the case of a single pool, the mechanisms regulating spontaneous and evoked neurotransmitter release differ. For example, removal of membrane cholesterol has been shown to enhance spontaneous exocytosis while suppressing evoked neurotransmitter release in both central synapses and NMJs [122-126].

Interestingly, in glutamatergic hippocampal synapses, spontaneous neurotransmitter release can activate receptors on the postsynaptic membrane that remain unresponsive during evoked release [127]. Inhibitory synapses exhibit two distinct receptor populations: one of them responds to both evoked and spontaneous release, while other is activated exclusively by the evoked neurotransmitter release. This segregation of receptor activation may reflect the role of spontaneous neurotransmitter release in synapse maturation and sustaining communication in mature synapses [128].

Spontaneous release can occur through various mechanisms and can be categorized into the following types:

I. Ca^{2+}-independent spontaneous exocytosis that involves random fusion of docked vesicles. In this scenario, RRP vesicles released in response to the stimulation fuse spontaneously via random interactions upon overcoming the energy barrier [70]. Because this process involves RRP vesicles, the molecular machinery mediating it is largely the same as that

of the evoked release. Recent studies in hippocampal neurons showed that the frequency of spontaneous exocytosis is influenced by the structure and spatial distribution of vesicles in the nerve terminal. Within the framework of this concept, the reserve pool imposes geometric constraints on the volume occupied by more mobile recycling pool vesicles. As these vesicles attempt to occupy a larger volume, they generate an entropic force that exerts pressure on RRP vesicles, thereby enhancing spontaneous exocytosis [129].

II. Ca^{2+}-dependent spontaneous exocytosis can result from (i) random opening of Ca^{2+} channels in the plasma membrane [70] (for example, selective blockers of voltage-gated Ca^{2+} channels suppress spontaneous exocytosis in NMJs [130, 131]) or (ii) Ca^{2+} release from intracellular stores [132]. Stochastic fluctuations in intracellular Ca^{2+} levels can trigger the fusion of docked and undocked vesicles. It has been shown that spontaneous and evoked releases are triggered by different Ca^{2+} sensors. In particular, synaptotagmin 1 and 2, located on SVs, contribute to the synchronous release. Doc2, located in the cytoplasm, and synaptotagmin 7, located on the presynaptic membrane, are more sensitive to Ca^{2+} concentrations and trigger spontaneous exocytosis upon small fluctuations in the Ca^{2+} levels. Moreover, the influx of Ca^{2+} through different calcium channels initiates different types of neurotransmitter release. Voltage-gated Ca^{2+} channels primarily trigger spontaneous release, while TRPV1 channels contribute to the asynchronous release [133]. In NMJs, reactive oxygen species (ROS)-dependent activation of TRPV1 channels can increase either asynchronous or spontaneous release, depending on the primary ROS source (mitochondria or NADPH oxidase) [124, 134]. L-type voltage-gated Ca^{2+} channels can enhance both spontaneous and asynchronous release, as observed in frog NMJs [135]. These findings suggest that exocytosis sites may be spatially organized and more adapted to different modes of neurotransmitter release. Such sites likely vary in their composition of calcium channels and signaling proteins, as well as their proximity to the endoplasmic reticulum and mitochondria.

A number of studies show that some vesicles are more prone to spontaneous fusion than others. This difference can be attributed to the different molecular machinery involved in spontaneous versus evoked release. For example, SNARE proteins VAMP7, VAMP4, and vti1a are primarily responsible for spontaneous release, whereas synaptobrevin 1 and 2 mediate evoked release [33, 107].

These proteins are distributed across vesicles in varying proportions. Vesicles enriched in proteins linked to spontaneous release are more likely to undergo spontaneous fusion and are thus identified as part of the spontaneous pool. Vesicles containing a mixture of proteins for both release types can contribute to both spontaneous and evoked release with a comparable probability.

ALTERATIONS IN THE STRUCTURE OF VESICULE POOLS IN NEUROLOGICAL DISORDERS

Parkinson's disease. Changes in the structure of SV pools are among the earliest manifestations of Parkinson's disease, occurring even before the formation of α-synuclein aggregates that ultimately cause the death of dopaminergic neurons. Overexpression of α-synuclein leads to a significant increase in the reserve pool, promoting the clumping of SVs [10, 11]. This overexpression also disrupts the mobilization of SVs from the reserve pool to the RRP, as well as their priming and endocytosis. The recycling pool diminishes, the number of fusion-ready vesicles decreases, and SVs are redistributed further from the AZ. Collectively, these alterations weaken neurotransmission [136, 137].

Injection of an antibody targeting the N-terminal domain of α-synuclein into lamprey reticulospinal synapses reduced the number of SVs in both the reserve pool and those docked at the AZ and caused dispersion of large SV clusters into smaller ones [138]. Deletion of α-, β-, and γ-synucleins suppressed SV inter-linking by short connectors (as observed by cryo-electron tomography), although the number of AZ-attached vesicles increased [139]. Parkinson's disease-associated mutations in the α-synuclein gene inhibited endocytosis and impaired replenishment of the RRP, as was shown in calyx of Held terminals [140]. Likewise, α-synuclein deletion reduced the reserve pool size and its contribution to the RRP replenishment, suppressing neurotransmission during prolonged hippocampal synaptic activity [141]. Furthermore, downregulation of genes encoding Parkinson's disease-related proteins LRRK2 (leucine-rich repeat kinase 2) and PINK1 impaired SV mobilization from the recycling and reserve pools, respectively [142, 143].

Therefore, alterations in the levels of α-synuclein or its mutations disrupt the normal organization of the reserve pool and its ability to restore the RRP during synaptic activity. As a result, evoked neurotransmitter release is significantly reduced even before the onset of neuronal death.

Alzheimer's disease. The prefibrillar amyloid variant $A\beta_{1\text{-}42}$, which forms at the earliest stages of Alzheimer's disease, impairs synaptic function in part by disrupting vesicle transport between synaptic boutons, thereby limiting synaptic plasticity. The mechanism is associated with the increased Ca^{2+} release from mitochondria, which triggers hyperphosphorylation of synapsin 1 and calcium/calmodulin-dependent

kinase IV (CAMKIV). This disrupts the SV transport between synapses, leading to the increased heterogeneity in the SV pool sizes across individual synapses [9]. Pharmacological activation of CYP46A1, a key enzyme controlling brain cholesterol turnover, reduced the phosphorylation of synaptic proteins and normalized the structure of SV pools in mice overproducing amyloid β peptide [144, 145].

A mutation in the gene encoding BIN1, a member of the BAR domain protein superfamily, has been linked to an increased risk of developing late-onset Alzheimer's disease. In BIN1 knockout mice, excitatory hippocampal synapses exhibit an increased number of docked vesicles, along with the expanded reserve pool, i.e., changes associated with the suppression of evoked exocytosis. A decrease in the activity-dependent neurotransmitter release and SV accumulation may contribute to early memory impairment [146].

An expansion of SV pools can lead to the increase in the frequency of spontaneous neurotransmitter release. It has been shown that amyloid β peptide promotes spontaneous exocytosis by increasing the number of SVs and release sites [129]. At the early disease stage, amyloid β peptide may upregulate the fraction of functionally active vesicles; however, concurrent disruption of the endocytotic recycling mechanism ultimately leads to synaptic deficit [147]. Amyloid β oligomers were found to cause an increase in the reserve pool size, while disrupting endocytosis and formation of fusion-competent SVs in cultured hippocampal neurons [148]. Over time (i.e., at the late disease stage), these alterations should lead to the depletion of the SV pool. Indeed, the density of SVs in cortical axons of patients with clinically diagnosed Alzheimer's disease was significantly reduced, mainly due to a diminished reserve pool [149].

Notably, at low physiological concentrations, amyloid β peptide (in particular, its isoforms $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}16}$) increases the size of the recycling pool through a mechanism associated with the stimulation of cholinergic signaling, activation of α7-nicotinic cholinergic receptors, and subsequent calcium/calcineurin-dependent dephosphorylation of synapsin 1 [150]. Consistent with this observation, early stages of Alzheimer's disease are characterized by an amyloid β-associated increase in the levels of SV-related proteins, expansion of the total SV pool, and presynaptic potentiation [151]. It should be noted that enlargement of the SV pool in the absence of impaired evoked exocytosis may enhance the learning capacity. The cohesin complex protein stromalin, previously identified as a memory suppressor, affects the anterograde transport of vesicles along the axon. Dopaminergic neurons of stromalin-knockout *Drosophila* flies exhibited an increase in the number of vesicles in the presynaptic terminals without changes in the number of synapses, axons, or neurons themselves. At the same time, mutant *Drosophila* flies demonstrated an improvement in the learning ability [152].

Hence, the physiological effects of low amyloid peptide concentrations may be associated with an increase in the sizes of the recycling/reserve pools. However, elevated amyloid peptide levels and associated pathological conditions disrupt SV recycling and promote accumulation of defective vesicles and excessive spontaneous exocytosis, which together disrupt communication between neurons.

Amyotrophic lateral sclerosis. Hyperexcitability of motor neurons contributes to their death due to excitotoxicity. An increase in the size of RRP in the spinal cord excitatory synapses and spontaneous release in NMJs were detected at the presymptomatic stage in hSOD1(G93A) mice [13, 14]. At the same time, both the density of vesicles in the RRP and the size of AZs in the inhibitory nerve terminals on motor neurons of these animals were reduced at the pre- and early symptomatic stages. Inhibition of NO synthase mitigated these alterations in inhibitory synapses [153]. As the disease progressed to the symptomatic stage, NMJs exhibited further dysfunction characterized by the increased NO production, suppression of synaptic vesicle mobilization, and diminished SV pool [154, 155].

ALS can be caused by mutations disrupting the nuclear localization of the RNA-binding protein FUS. These mutations induce FUS relocation to vesicles of the reserve pool [156]. In mutant mice, synapsin 1 expression is upregulated, and synaptic vesicle recruitment to exocytosis during periods of intense activity at NMJs is suppressed [157].

Therefore, hyperactivation of motor neurons due to a decrease in the RRP in inhibitory synapses, alongside an increase in the RRP in excitatory synapses of the spinal cord and suppression of SV mobilization in NMJs, may contribute to the progression of ALS.

Schizophrenia is a polyetiological disease with multiple contributing factors. Among these is a mutation in a gene coding for the dystrobrevin-binding protein dysbindin, which is associated with a reduced RRP size and disruption in its replenishment during high-frequency activity. In dysbindin-knockout mice, these alterations cause deficits in synaptic transmission, which, in turn, can lead to various mental disorders [12, 158, 159].

Memory disorders. In *Drosophila melanogaster*, a single session of aversive olfactory training produces two distinct forms of memory: labile memory, which decays within a few hours and is easily disrupted after shock exposure, and stable (consolidated) memory, which persists long term and resists to amnestic interference. These memory types are supported by functionally distinct SV pools in the presynaptic

terminal, and perturbations in their dynamics can lead to memory impairment. Labile memory is associated with changes in the reserve pool. Exposure to stressors (cold or mechanical shock) disrupt this pool due by causing synapsin dispersion, which ultimately leads to retrograde amnesia. In contrast, the formation of consolidated memory in *D. melanogaster* critically depends on the AZ protein Rab3, which regulates recruitment of SVs to the AZ and their priming. Rab3 modulates the size of the RRP and the probability of vesicle release – the key determinants of synaptic plasticity [160].

Noonan syndrome belongs to the group of Rasopathies, which are characterized by disruptions in the Ras–MAPK signaling cascade. This disease is associated with mutations in the gene of protein tyrosine phosphatase PTPN11 and is manifested by the developmental delay and intellectual disability. Mutations in the *PTPN11* gene lead to presynaptic alterations, including a decrease in the number of RRP vesicles and in the total number of vesicles. In addition, both the probability of exocytosis and the rate of synaptic vesicle endocytosis are diminished [161].

Mitochondrial dysfunction. Mutations in the gene of the dynamin-related protein 1 (DRP1), a key mediator of mitochondrial division, lead to neurodegenerative changes. In addition to disruptions in mitochondrial remodeling, selective deletion of the DRP1-encoding gene in the presynaptic terminals of calyx of Held synapses caused changes in the SV clustering, as well as a decrease in the size of the RRP, its replenishment, and recycling, which were accompanied by a reduction in the presynaptic terminal volume [162]. Disruption of mitochondrial fusion by knocking down the neuronal protein mitofusin 2 also reduced the levels of SV proteins and impaired recruitment of SVs from the reserve pool to the RRP during prolonged stimulation in hippocampal axons [163].

EFFECTS OF BIOLOGICALLY ACTIVE SUBSTANCES ON SYNAPTIC VESICLE POOLS

Cannabinoids. In addition to their inhibitory action on Ca^{2+} channels in the AZ through activation of CB1 receptors and reduction of neurotransmitter release, cannabinoids modulate long-term plasticity by affecting the size of vesicle pools. Activation of the G protein-coupled CB1 receptor decreases cAMP levels and protein kinase A (PKA) activity. Suppression of the PKA-mediated phosphorylation of synapsin 1 enhances its binding to synaptic vesicles, thus shrinking the superpool. As a result, spontaneous exocytosis decreases while the reserve pool expands, providing a larger reservoir of vesicles available for release during subsequent high-frequency stimulation [164]. Activation of CB1 receptors leads to the redistribution of SVs in presynaptic boutons: vesicle clustering increases and the number of vesicles in the AZ decreases, indicating a transition of RRP vesicles into the reserve pool. These changes, which depend on the actomyosin cytoskeleton and Rho-associated kinase signaling, lead to reduced activity at the corticostriatal synapses [165]. Activation of presynaptic CB1 receptors in excitatory synapses in motor neurons decreases the RRP size, reducing synaptic transmission [166].

Reactive oxygen species enhance spontaneous release of vesicles, which otherwise are released in response to stimulation under normal conditions. This phenomenon has been documented in neurodegenerative diseases [167]. Optogenetically induced ROS production by presynaptic mitochondria in *Drosophila* NMJs increased both spontaneous release and the number of docking sites for RRP vesicles [168]. However, the application of exogenous H_2O_2 and pro-oxidants suppressed evoked neurotransmitter release and inhibited mobilization of synaptic vesicles in NMJs [169], while endogenous stimulation of ROS production had the opposite effect, promoting vesicle mobilization from the reserve pool [170-173].

Zinc ions are concentrated in a subset of ZnT3 transporter-expressing SVs in glutamatergic and GABAergic synapses [174, 175]. These vesicles undergo recycling with the involvement of the adaptor protein AP-3 [176, 177], which mediates SV formation from endosomes and is primarily associated with asynchronous neurotransmitter release [178]. A subpopulation of vesicles containing Zn^{2+} releases neurotransmitters predominantly during high-frequency stimulation [175]. At low nanomolar concentrations, Zn^{2+} suppresses the mobilization of vesicles from the reserve pool during high-frequency stimulation and reduces the size of the RRP, which correlates with a decrease in evoked synchronous exocytosis and spontaneous exocytosis in NMJs [179]. Hypothetically, low Zn^{2+} concentrations can act as regulators of the RRP size and SV mobilization through a negative feedback mechanism, thus limiting neurotransmission. Although the presence of ZnT3 in SVs in motor nerve terminals has not been reported so far, the main source of free Zn^{2+} may be active muscle fibers.

At high micromolar concentrations, Zn^{2+} enhances spontaneous neurotransmitter release in NMJs and central synapses [180, 181]. This effect may result from upregulated SV docking in the AZ due to the increased ability of vesicular synaptotagmin 1 to bind to anionic lipids of the AZ membrane in the presence of Zn^{2+} [181]. Interestingly, Zn^{2+} protects central and peripheral synapses from the toxic effects of Cd^{2+}, one of the most common pollutants [182, 183].

Atorvastatin, an inhibitor of cholesterol biosynthesis, suppresses neurotransmitter release during periods of intense activity by reducing SV mobilization from the reserve pool, which is associated with the upregulation of synapsin 1 in motor nerve terminals and decrease in the population of SVs participating in exo- and endocytosis [8]. One possible explanation is that retention of a greater number of SVs in the "inactive" pool helps preserve cholesterol reserves in vesicle membranes during cholesterol-lowering therapy. At the same time, NMJs exhibit an increase in the activity-dependent uptake of low-density lipoproteins (cholesterol carriers), suggesting activation of bulk endocytosis [184]. Notably, oxidized cholesterol derivatives at submicromolar concentrations can bidirectionally regulate the size of the SV pool engaged in exo- and endocytosis in NMJs [185-187]. One potential mechanism of their action is modulation of biophysical properties of synaptic membranes.

Fluoxetine, a selective serotonin reuptake inhibitor that increases serotonin levels in the synaptic cleft, also modulates the distribution of SVs between the vesicle pools. During high-frequency stimulation, fluoxetine promotes the mobilization of vesicles from the reserve pool to the recycling pool, leading to the increase in the probability of evoked exocytosis. Hence, the antidepressant effects of fluoxetine may also be associated with the enhanced vesicle cycling. Additionally, fluoxetine has been shown to increase spontaneous neurotransmitter release, accompanied by activation of serotonin receptors [7].

Ketone bodies. The ketogenic diet – a low-carbohydrate, high-fat regimen that induces ketosis through hepatic production of ketone bodies (acetoacetate, β-hydroxybutyrate) – is an established treatment for drug-resistant childhood epilepsy. Ketone bodies modulate gene expression via epigenetic mechanisms, including β-hydroxybutyrylation and acetylation of histones. This leads to changes in the expression of synaptic genes in the hippocampus and remodeling of hippocampal synapses. The ketogenic diet reduces the size of the RRP, resulting in a decreased glutamate release during high-frequency stimulation, which suppresses synaptic potentiation and shifts the short-term plasticity toward depression. Collectively, these changes reduce hippocampal neuronal excitability, which is thought to underlie the antiepileptic effect of ketogenic diet [188].

ORGANIZATION OF SYNAPTIC VESICLE POOLS IN INDIVIDUAL SYNAPSES

Ribbon synapses are predominantly found in structures involved in the analysis of visual and auditory information, specifically, in auditory inner and outer hair cells, bipolar cells, photoreceptors, and pinealocytes. They are characterized by a special organization of vesicle pools that facilitates rapid and efficient transmission of visual and auditory signals [189, 190].

A defining feature of ribbon synapses is the presence in the AZ of synaptic ribbons, proteinaceous organelle formed mainly from the Ribeye protein, that tether the vesicles. Based on location, these vesicles classified as membrane-proximal, and ribbon-associated (RA) or membrane-distal. In addition, a population of undocked vesicles is freely distributed in the cytoplasm. Membrane-proximal vesicles reside at the base of synaptic ribbons; they are docked to the presynaptic membrane and represent functional analogs of RRP vesicles [191]. A high density of voltage-gated calcium channels is observed near the synaptic ribbons, facilitating rapid and synchronous fusion of these vesicles [192].

RA vesicles are positioned slightly farther from the plasma membrane yet remain tethered to synaptic ribbons. When the pool of membrane-proximal vesicles becomes depleted, RA vesicles rapidly replenish it. The presence of synaptic ribbons allows these vesicles to quickly form a fusion-ready SNARE complex, ensuring the passage through all ATP-dependent steps more efficiently, thus shortening the time for RRP replenishment [191, 193].

Vesicles dispersed in the cytoplasm represent an analog of the reserve pool [194]. However, unlike the classical reserve pool, these vesicles are not associated with synapsin, which increases their mobility and allows to quickly replenish the pool of vesicles associated with synaptic ribbons [195].

Together, these three vesicle pools support sequential phases of neurotransmitter release at ribbon synapses. Membrane-proximal vesicles mediate the fast (phasic) component of release; RA vesicles sustain tonic neurotransmitter release during continuous stimulation; and cytoplasmic vesicles maintain neurotransmission during prolonged stimulation, contributing to the third, slower component [195].

Vesicle exocytosis can occur at AZs either associated or not associated with synaptic ribbons. In the latter case, cytoplasmic vesicles provide neurotransmitter release triggered by the calcium efflux from the endoplasmic reticulum [196]. Interestingly, this mechanism has been found only in rods, where release from AZs not associated with synaptic ribbons maintains basal glutamate levels in the synaptic cleft [194].

Synaptic ribbons facilitate rapid vesicles cycling [190]. During stimulation, mobile vesicles are recruited and docked to synaptic ribbons, enabling continuous and complete replacement of released RRP vesicles. The presence of the RRP allows vesicles

to be released synchronously long after the onset of stimulation [191].

Spontaneous neurotransmitter release in ribbon synapses can be either Ca^{2+}-dependent or Ca^{2+}-independent [197]. In rods, both mechanisms are present, whereas in cones, neurotransmitter release is predominantly Ca^{2+}-independent [194]. Ca^{2+}-dependent vesicle fusion occurs mainly at AZs associated with synaptic ribbons, likely due to the high density of voltage-gated calcium channels in these regions. In rods, by contrast, Ca^{2+}-independent spontaneous release has been observed at AZs not associated with synaptic ribbons [197].

Another distinctive feature of ribbon synapses is that the transmitted information is encoded not only by the stimulus frequency but also by its amplitude. In other words, stronger stimuli lead to the release of greater amounts of neurotransmitter, resulting in larger amplitudes of postsynaptic responses [198]. Multivesicular release, which can vary in amplitude depending on how many vesicles are released at the same time, enables the transmission of an ultra-rapidly changing signal to the postsynapse within milliseconds. In contrast, frequency-based encoding through the exocytosis of single vesicles operates on a slower timescale (seconds), strongly delaying afferent signals. Consequently, this mode of release is better suited for encoding sustained, stable stimuli [199].

Central small (hippocampal) synapses. Hippocampal synaptic boutons are small and contain ~100-400 vesicles, yet they provide synaptic transmission at a frequency of 10-100 Hz [200, 201]. Maintaining such high-frequency activity requires efficient organization and recycling of SVs.

Hippocampal synapses contain the RRP, the superpool, and the recycling, reserve, and resting pools. RRP vesicles are docked to the plasma membrane. Upon stimulation, these vesicles undergo cycles of exo- and endocytosis and become part of the recycling pool, which sustains synaptic activity for long periods of time [87]. RRP vesicles in the hippocampus are replenished through clathrin-mediated endocytosis, kiss-and-run mechanism, and ultrafast endocytosis [16, 49, 50]. Although RRP vesicles in the hippocampus are predominantly released in a synchronous manner [41, 66-68], they also contribute to spontaneous neurotransmitter release [71-74].

Reserve pool vesicles constitute a substantial fraction of the total SV population. During prolonged neuronal activity, they sustain neurotransmission by undergoing exocytosis. However, vesicle release from the reserve pool is predominantly asynchronous, as these vesicles require additional time for mobilization, docking, and fusion [89]. The reserve pool in the hippocampal synapses can be further divided into two distinct subpopulations: fast- and slow-mobilizing vesicles. These subpools participate in exocytotic and endocytotic cycles in parallel and remain functionally separate, even under high-frequency stimulation [104]. The reserve pool is replenished primarily through bulk endocytosis [52]. A considerable fraction of synaptic vesicles in hippocampal synapses remains inactive *in vivo*, forming the resting pool [118]. These vesicles may play a role in modulating spontaneous neurotransmitter release [129].

The relative proportions of the RRP and reserve pool vary across different synaptic boutons in the hippocampus. Changes in the size of vesicle pools play an important role in the formation of synaptic plasticity. The redistribution of vesicles between boutons is provided by the superpool [5].

Calyx of Held synapses are giant glutamatergic synapses in the auditory brainstem. The total number of vesicles in the presynaptic terminal reaches 200,000-300,000 [117], and the number of AZs is 300-700 [202].

The RRP in this synapse is heterogeneous and can be divided into fast- and slow-releasing components based on the probability of exocytosis (Pr). Vesicles with high Pr provide synchronous release in response to a single action potential, while vesicles with low Pr are not released during low-frequency activity and ensure asynchronous neurotransmitter release after tetanic stimulation [203]. The heterogeneity of RRP vesicle exocytosis is largely attributed to differences in the sensitivity to cytosolic Ca^{2+} [24]. Some studies confirm the intrinsic heterogeneity in Ca^{2+} sensitivity among vesicles themselves [25], while others emphasize positional heterogeneity of vesicles located at different distances from calcium channels [204].

AZs in calyx of Held synapses exhibit substantial variability in the number of voltage-gated calcium channels, ranging from 5 to 200 per AZ. This variability likely contributes to differences in local Ca^{2+} dynamics and, consequently, to diverse Pr values observed among RRP vesicles [31].

Most vesicles (~80%) in the calyx of Held synapses participate in the cycles of exocytosis and endocytosis [4]. The recycling pool is replenished primarily through mobilization of vesicles from the reserve pool [44, 46], while newly endocytosed vesicles are preferentially routed to the reserve pool [46]. During moderate-frequency stimulation, vesicle retrieval is predominantly mediated by clathrin-dependent endocytosis. At higher stimulation frequencies, faster mechanisms, such as kiss-and-run and bulk endocytosis, become more prominent [84]. The resting pool comprises roughly 20% of the total vesicle population and is not released even under high-frequency stimulation [117].

***Drosophila* neuromuscular junction** contains ~84,000 vesicles. The RRP constitutes 14-19% of the total vesicle pool [205]. A distinctive feature of the spatial organization of vesicle pools in *Drosophila* NMJ is that the RRP is positioned at the periphery of the nerve terminal, whereas the reserve pool occupies its central region [206]. During low-frequency stimulation, RRP vesicles undergo continuous recycling to sustain basal activity levels. In contrast, the reserve pool is recruited into exocytosis and endocytosis cycling primarily during periods of high-frequency activity [206, 207]. Replenishment of SV pools is mediated by several clathrin- and dynamin-dependent endocytosis mechanisms [208].

The homeostatic plasticity at *Drosophila* NMJs is manifested as a compensatory increase in neurotransmitter release in response to the reduced postsynaptic receptor activity. A key mechanism underlying presynaptic homeostatic plasticity is an expansion of the RRP [209, 210].

Vesicles involved in evoked exocytosis are also involved in spontaneous exocytosis. However, *Drosophila* NMJ may contain a subset of vesicles specialized for spontaneous neurotransmission [211]. Although the same AZs can support both evoked and spontaneous release, functional heterogeneity exists, as some AZs preferentially mediate evoked exocytosis, whereas others are more strongly associated with spontaneous neurotransmitter release [212, 213].

Frog neuromuscular junction. Frog NMJ contains hundreds of thousands of vesicles [2]. Approximately 20% of these vesicles belong to the RRP, while the remaining form the reserve pool [20]. In frog NMJs, the RRP and reserve pools operate in parallel way without overlapping [85], and distinct exocytosis sites likely mediate release from each pool.

The RRP is distributed throughout the nerve terminal [20]. During low-frequency activity, RRP vesicles can be recycled for extended periods of time without involvement of the reserve pool, as they are replenished via endocytosis. Upon high-frequency stimulation (30 Hz), the RRP becomes depleted after 10-20 s, but is fully recovered within 1 min through endocytosis. Reserve pool vesicles do not contribute to the RRP replenishment [85].

The reserve pool participates in synaptic transmission during high-frequency stimulation (20-30 Hz) and undergoes recycling in parallel with the RRP, as vesicles retrieved by endocytosis are returned to the reserve pool [85]. The reserve pool is replenished primarily through bulk endocytosis [108]. After endocytosis, vesicles originating from the two pools remain segregated and do not mix [17].

At the NMJ, the same vesicles participate in both synchronous and asynchronous exocytosis [214]. Different subtypes of Ca^{2+} channels may be preferentially involved in these two forms of release [134, 135]. Spontaneous release of vesicles is Ca^{2+}-dependent and is driven by both intracellular Ca^{2+} [215] and exogenous Ca^{2+} entering through Ca^{2+} channels of the plasma membrane (e.g., L-type channels) [130, 135]. Depolarization can also enhance spontaneous exocytosis in a Ca^{2+}-independent manner [216].

Mouse neuromuscular junction. Mouse motor nerve terminals contain approximately 400-850 AZs, with 1-2 vesicles per each AZ. Accordingly, the RRP comprises ~800-1700 vesicles, with the total vesicle population in the NMJ exceeding 400,000-800,000. Vesicle participation in exocytosis strongly depends on the stimulation frequency. At low frequencies, only a subset of docked RRP vesicles undergoes exocytosis and recycling, whereas high-frequency activity recruits nearly entire RRP [22, 24, 217].

Mouse NMJs exhibit pronounced frequency-dependent mobilization of SVs. Thus, mouse nerve terminals contain a “housekeeping” vesicle pool that equally sustains neurotransmission upon both low- and high-frequency stimulation. However, efficient transmission during high-frequency activity requires recruitment of an additional “plug-in” pool that remains largely inactive at low frequencies [218]. The contribution of this auxiliary pool is enhanced by activation of β2-adrenergic receptors [219].

The type of endocytosis used for the pool replenishment also depends on the stimulation frequency. At moderate frequencies, exocytosis primarily occurs via full fusion of vesicles with the presynaptic membrane. Under high-frequency conditions, faster recycling mechanisms, such as kiss-and-run, become more prominent [56, 220]. After high-frequency stimulation, vesicle retrieval occurs through both clathrin-dependent and clathrin-independent endocytosis [110, 220, 221], while bulk endocytosis is activated during intense stimulation of the motor nerve [110, 222].

Reserve pool vesicles participate in both synchronous and asynchronous neurotransmitter release [99, 105]. Evidence suggests that largely overlapping vesicle populations participate in both evoked and spontaneous exocytosis [223].

CONCLUSION

Efficient synaptic transmission relies on continuous recycling of vesicles from functionally different pools. RRP vesicles are primed for fusion with the presynaptic membrane, enabling rapid, synchronous neurotransmitter release in response to stimulation. The recycling pool sustains transmission during moderate stimulation and short periods of high-frequency stimulation, whereas the reserve pool supports

prolonged neurotransmission when recycling vesicle pool becomes depleted. In addition, a subset of vesicles serves as a reservoir of lipids and proteins, ensuring a rapid supply of essential components for synaptic function. The superpool provides the redistribution of vesicles between active and inactive synaptic boutons, while the spontaneous pool contributes to synapse maturation and maintenance of baseline activity.

Differences in the cycling of vesicles belonging to different pools enable synapses to adapt to varying levels of activity. Under resting conditions and upon moderate stimulation, vesicle replenishment occurs primarily via clathrin-mediated endocytosis. During high-frequency stimulation, faster mechanisms, such as kiss-and-run and ultrafast endocytosis, allow vesicle pool recovery in a short period of time. Bulk endocytosis preserves presynaptic membrane integrity during periods of intense exocytosis.

The presence in presynaptic terminals of functionally heterogeneous populations of vesicles, differing in their capacity for exocytosis, mobilization, and recycling kinetics, together with the existence of multiple modes of exo- and endocytosis, provides a fundamental basis for synaptic plasticity and adaptation of the synaptic apparatus.

The importance of these mechanisms is emphasized by the fact that disruptions in the vesicle cycling and organization of SV pools contribute significantly to the pathogenesis of numerous neurological and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, schizophrenia, and ALS. Moreover, SV dynamics and recycling mechanisms represent targets for various pharmacological agents, such as fluoxetine and atorvastatin.

Further investigation into the organization and functional properties of SV pools is essential for a comprehensive understanding of neuronal communication under both physiological and pathological conditions. In particular, the mechanisms governing the formation and reorganization of vesicle pools constitute promising targets for therapeutic intervention. However, the processes underlying the genesis of SV pools remain largely unexplored.

Unresolved fundamental questions remain: which molecular mechanisms determine the assignment of SVs to specific pools; how the organization of these pools is preserved despite repeated reuse of the same vesicles; which regulatory systems govern the pool size and mediate vesicle exchange between the pools; and what underlies the heterogeneity within individual pools, particularly the RRP and reserve pool. It is also unclear how SV pools are altered under pathological conditions associated with mitochondrial dysfunction, endoplasmic reticulum stress, impaired autophagy, and disruptions of the endolysosomal system. Given that SVs are composed of substantial amounts of proteins and lipids and undergo continuous cycles of exocytosis and endocytosis, the functioning of SV pools is likely tightly coupled to cellular metabolic processes. Elucidating these relationships could substantially advance our understanding of the global interplay between metabolism and synaptic function. Moreover, identifying mechanisms that regulate the behavior of distinct vesicle pools and the rate of SV recycling may suggest new therapeutic strategies for a broad range of neurological and neurodegenerative disorder

Abbreviations

AZ	active zone
NMJ	neuromuscular junction
RRP	readily releasable pool
SV	synaptic vesicle

Acknowledgments

The authors are grateful to A. N. Tsentsevitsky (Kazan Institute of Biochemistry and Biophysics) for valuable comments and discussion.

Contributions

A.M.P. developed the study concept and edited the manuscript; C.R.G. wrote and edited the text of the article.

Funding

This publication was supported by the Brain Program of the Idea Scientific Center for Advanced Interdisciplinary Research and State Assignment for the Kazan Scientific Center.

Ethics approval and consent to participate

This work does not contain studies involving human or animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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