The mechanisms and functions of spontaneous neurotransmitter release

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Abstract | Fast synaptic communication in the brain requires synchronous vesicle fusion that is evoked by action potential-induced Ca^{2+} influx. However, synaptic terminals also release neurotransmitters by spontaneous vesicle fusion, which is independent of presynaptic action potentials. A functional role for spontaneous neurotransmitter release events in the regulation of synaptic plasticity and homeostasis, as well as the regulation of certain behaviours, has been reported. In addition, there is evidence that the presynaptic mechanisms underlying spontaneous release of neurotransmitters and their postsynaptic targets are segregated from those of evoked neurotransmission. These findings challenge current assumptions about neuronal signalling and neurotransmission, as they indicate that spontaneous neurotransmission has an autonomous role in interneuronal communication that is distinct from that of evoked release.

Docked vesicles
Synaptic vesicles that are tethered to the presynaptic membrane or the active zone structure. According to current views, not all docked vesicles are fully primed for fusion and release of neurotransmitter.

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Our current insights into the mechanisms underlying synaptic transmission originate from experiments that were conducted in the 1950s by Bernard Katz and colleagues[1–3] (FIG. 1). A key aspect of these studies was the discovery of spontaneous neurotransmitter release events, which seemed to occur in discrete 'quantal' packets (FIG. 2). This fundamental observation enabled the complex and seemingly intractable nature of action potential-evoked neurotransmission to be analysed and understood on the basis of its unitary components[2–4]. Although the original work solely relied on electrophysiological analysis, later studies that used electron microscopy provided visual validation of the hypothesis that neurotransmission occurs through fusion of discrete synaptic vesicles that contain neurotransmitters with the presynaptic plasma membrane[5,6].

The quantal hypothesis of neurotransmission now has overwhelming experimental support, and we are beginning to understand the exquisite molecular mechanisms involved[7]. However, the discovery of the molecular machinery that enables presynaptic vesicle fusion to occur also uncovered some unexpected distinctions between the processes that lead to spontaneous and action potential-evoked neurotransmitter release. Early studies that used clostridial toxins to impair presynaptic machinery components[8,9] and later work that used genetic manipulations to selectively knock out the function of individual fusion proteins showed varying degrees of presynaptic release impairment; however, in most circumstances, the process of spontaneous neurotransmitter release was left relatively intact[10–13]. Thus, although these experiments proved the vesicular hypothesis of neurotransmitter release, they raised the question of whether spontaneous release events originate from the same vesicular trafficking pathway as evoked neurotransmission[14]. Recent advances in our understanding support the autonomous nature of spontaneous neurotransmission and indicate its key role in the signalling that leads to synaptic maturation and homeostasis. This Review presents an overview of the experimental results and conceptual developments that have given rise to this revised outlook on the mechanisms and functions of spontaneous neurotransmitter release.

Spontaneous release mechanisms

Specific synaptic vesicle fusion machinery. The traditional view of spontaneous neurotransmitter release posits that these spontaneous events occur randomly in the absence of stimuli owing to low-probability conformational changes in the vesicle fusion machinery[15]. Random fluctuations of the vesicle fusion machinery can be augmented in response to subthreshold elevations in presynaptic Ca^{2+} levels, which facilitate neurotransmitter release when the neuron is at rest[16,17]. Spontaneous events were initially thought to arise from fusion of the same docked vesicles and primed vesicles that mediate release after the arrival of a presynaptic action potential (called the readily releasable pool (RRP)). Indeed, several electrophysiological and presynaptic optical imaging experiments have documented a correlation between the responsiveness of evoked release events and that of

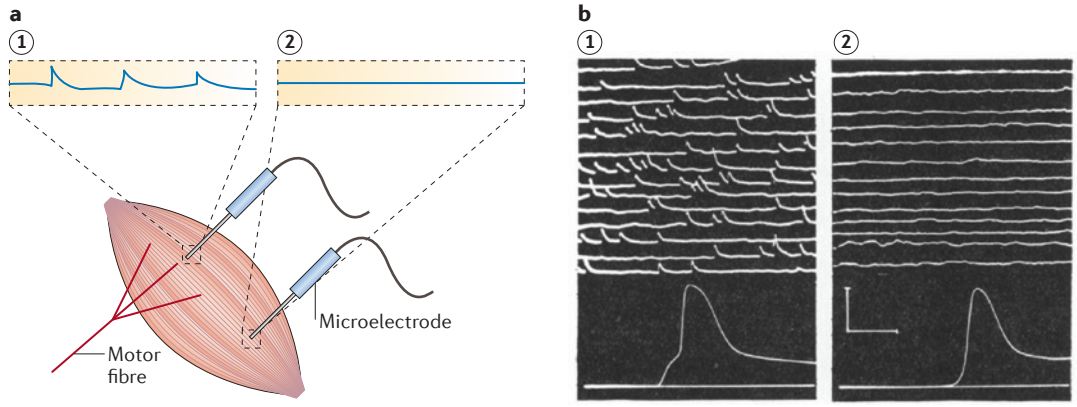


Figure 1 | **The earliest recordings of spontaneous synaptic activity. a** | The neuromuscular junction preparation used by Bernard Katz and colleagues, in which they initially described spontaneous cholinergic neurotransmitter release events, is shown. **b** | Intracellular recordings near the sites of nerve innervation (1), but not recordings at distal regions (2), showed random spontaneous voltage fluctuations that were sensitive to cholinergic agents, nerve denervation and osmotic pressure[2]. Part **b** reproduced from Fatt, P. & Katz, B. Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* 117, 109–128 (1952). John Wiley & Sons.

spontaneous release to presynaptic Ca^{2+} concentrations at the macroscopic level, which provides quantitative justification for their emergence from the same process[18,19]. However, an increasing number of molecular studies have identified mechanisms that either promote or suppress spontaneous release that are dissociable from their impact on evoked release[20,21].

In the brain, the canonical synaptic vesicular SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) protein synaptobrevin 2 (also known as vesicle-associated membrane protein 2 (VAMP2)) forms a complex with the plasma membrane SNARE synaptosomal-associated protein 25 (SNAP25) and syntaxin 1 to drive rapid action potential-evoked synaptic vesicle fusion[22]. Therefore, loss of synaptobrevin 2 in mice results in near-complete loss of the Ca^{2+}-dependent release that is evoked in tight synchrony with presynaptic action potentials. By contrast, a sizeable proportion of spontaneous neurotransmission and asynchronous release (which is loosely coupled to stimulation) are left intact in these mice[11,23]. This observation suggests that there may be a specialized role for alternative vesicular SNAREs in the maintenance of spontaneous and asynchronous neurotransmission.

Several additional SNAREs with structures similar to that of synaptobrevin 2 are expressed at low levels on synaptic vesicles. These include VAMP4, VAMP7 (also known as tetanus-insensitive VAMP) and VTI1A (vesicle transport through interaction with t-SNAREs homologue 1A)[24]. These vesicular SNAREs typically function in the fusion and trafficking of subcellular organelles within the neuron and possibly guide the biogenesis of synaptic vesicles through the secretory pathway[25,26]. However, there is also evidence that these alternative SNAREs are not homogeneously distributed among synaptic vesicle populations[25,27,28]. This molecular diversity suggests that some synaptic vesicles that drive spontaneous neurotransmission are likely to diverge from the classical synaptobrevin 2-dominant SNARE composition and comprise distinct populations. Furthermore, these SNAREs may be involved in the formation of alternative fusion complexes with specialized functional properties that enable them to selectively mediate spontaneous or asynchronous neurotransmission[29] (BOX 1).

Recent studies have examined the synaptic function of these alternative SNAREs and have uncovered their complementary roles to synaptobrevin 2 in synaptic transmission. In neurons, VTI1A is localized to cell bodies, where it is involved in the endosome and *trans*-Golgi network, as well as to presynaptic terminals, where a splice variant of VTI1A is enriched in purified synaptic vesicles[30]. VAMP7 is expressed throughout the adult brain. It is typically found in the cell body and dendrites of neurons but has also been shown to be present in presynaptic terminals in certain brain areas, such as the hippocampal dentate gyrus[31,32]. Simultaneous optical imaging of synaptobrevin 2 and VTI1A or VAMP7, as well as electrophysiological experiments, have shown that although both VTI1A and VAMP7 could be slowly mobilized in response to strong stimulation, VTI1A was preferentially trafficked under resting conditions[27]. Further experiments showed that gain or loss of function of VTI1A alters the regulation of a high-frequency spontaneous release event. These results support the hypothesis that VTI1A selectively maintains spontaneous neurotransmitter release[27].

Recent evidence also indicates a key role for VAMP7 in the regulation of spontaneous release. Earlier studies showed that VAMP7-containing synaptic vesicles are responsive to strong stimulation and may therefore perform asynchronous neurotransmission and spontaneous release[28,31]. A newer study demonstrated that although VAMP7 in its native form did not show much trafficking under resting conditions, VAMP7-enriched vesicles could be swiftly mobilized by the small increases in presynaptic Ca^{2+} concentration that occur in response to neuromodulators, such as the secreted glycoprotein reelin[33]. These findings are also consistent with experiments that took advantage of a membrane-binding

Primed vesicles
Vesicles that are docked and that have advanced through all the necessary molecular rearrangements of the SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) fusion machinery; that is, vesicles waiting for the influx of Ca^{2+} ions to trigger fusion. According to the current view, vesicle priming requires partial or full assembly of the SNARE complex, as well as interaction of SNAREs with other key fusion proteins, such as MUNC18, MUNC13 and other components of the presynaptic active zone.

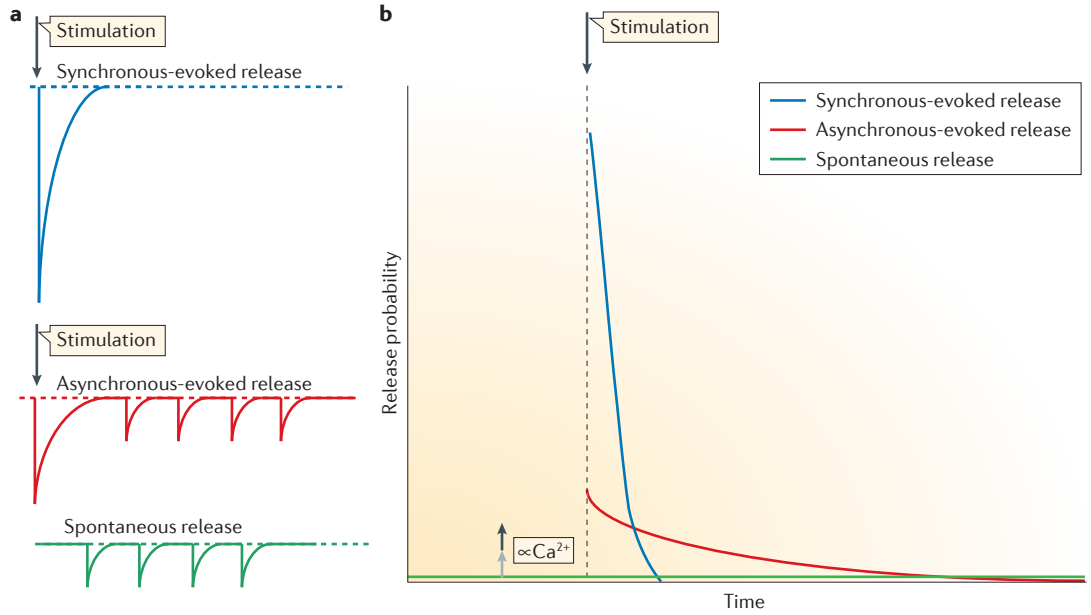


Figure 2 | **Three kinetically distinct forms of neurotransmitter release.** Three forms of neurotransmitter release with distinct Ca^{2+} dependence and time courses occur at the synapse. **a** | Graphical recording traces show representative examples of events that are detected in response to synchronous- and asynchronous-evoked release, and to spontaneous release during a typical electrophysiological experiment. **b** | The graph shows the relative time courses of decay in neurotransmitter release probability seen after presynaptic stimulation. Presynaptic action potentials and the resulting Ca^{2+} influx cause synchronous vesicle fusion within 1 ms. In some synapses, vesicle fusion is only loosely coupled to the timing of a presynaptic action potential and may thus outlast the duration of the action potential for 1 s or more, which leads to asynchronous neurotransmitter release. In addition, neurotransmitter release can occur spontaneously in the absence of presynaptic action potentials. However, the rate of such spontaneous release is proportional to intracellular Ca^{2+} levels.

fluorophore–cysteine–lysine–palmitoyl group (mCLING) to label recycling synaptic vesicles and image their molecular composition using super-resolution microscopy[25]. This approach revealed a relative enrichment of endosomal SNAREs on spontaneously recycling vesicles compared with their counterparts that trafficked during activity[25]. Taken together, these findings addressed a long-standing question by demonstrating a physiological role for synaptobrevin 2-independent spontaneous release events[11,23]. This work has also shown that these alternative vesicular SNAREs are molecular tags for independently functioning synaptic vesicle populations and provide a potential molecular basis for the selective regulation of spontaneous neurotransmitter release.

A recent study queried the synaptic function of another endosomal SNARE, VAMP4, and showed that, whereas synaptobrevin 2 is involved in rapid Ca^{2+}-dependent synchronous neurotransmission, VAMP4 selectively maintains bulk Ca^{2+}-dependent asynchronous release[34]. As indicated above, asynchronous release events are dependent on presynaptic action potentials, but their timing is only loosely coupled to the action potential stimulation[15]. In these experiments, VAMP4 did not show robust trafficking under resting conditions, although it was shown that VAMP4-enriched vesicles can respond to elevated presynaptic Ca^{2+} signals and promote release[33,34]. Accordingly, multicolour optical imaging of VAMP4 and synaptobrevin 2 trafficking at individual synapses revealed minimal overlap, which suggests that these two SNAREs traffic independently and maintain distinct forms of neurotransmission. These observations were also consistent with biochemical experiments that demonstrated that VAMP4 forms a stable complex with syntaxin 1 and SNAP25. However, unlike the synaptobrevin 2-driven complex, the VAMP4 complex did not readily interact with complexins or synaptotagmin 1 — proteins that are essential for fast synchronous neurotransmission[34]. This biochemical insight may explain why VAMP4 preferentially affects asynchronous release.

These experiments suggest that even the seemingly similar synchronous and asynchronous fusion events that occur at a single synapse can be diverse. Interestingly, this premise is consistent with experimental results obtained using fluorophore-assisted light inactivation of rod bipolar cell ribbons, which caused an acute reduction in the amplitude and frequency of asynchronous quantal events. However, after light inactivation of the ribbons, the initial evoked responses remained intact[35]. Along the same lines, at the *Caenorhabditis elegans* neuromuscular junction, distinct isoforms of the active zone protein Unc13 (which is the *C. elegans* homologue of mammalian MUNC13) affect the spatial segregation of synchronous and asynchronous release but do not alter spontaneous release[36].

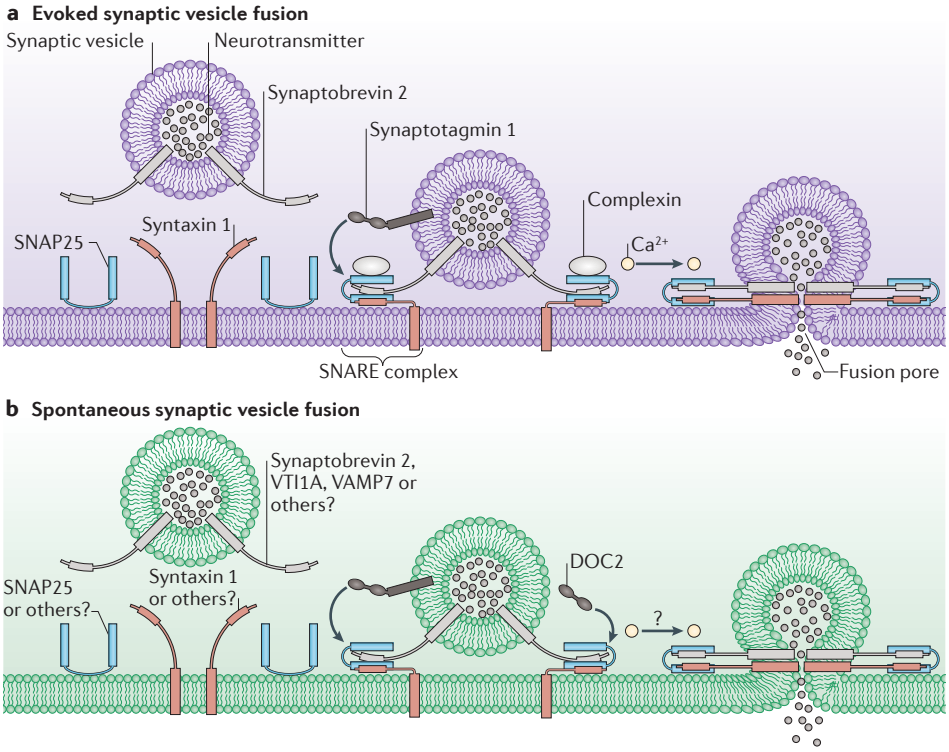
Despite the evidence that supports a key role for alternative SNAREs in spontaneous and asynchronous release processes, it is important to note that in mammalian central synapses a substantial proportion (70–90%)

Super-resolution microscopy
A form of light microscopy that achieves a spatial resolution of 50–100 nm, which is beyond the limit set by diffraction; it includes stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM).

Box 1 | **Divergent fusion machinery for spontaneous and evoked release**

The formation of a SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) complex by SNARE proteins — synaptobrevin 2, syntaxin 1 and synaptosomal-associated protein 25 (SNAP25) — mediates evoked synaptic vesicle fusion (see the figure, part **a**). Syntaxin 1 and synaptobrevin 2 are anchored on the plasma membrane and the synaptic vesicle membrane, respectively, by a transmembrane region, whereas SNAP25 is attached to the plasma membrane by palmitoylated cysteines. All SNAREs share a characteristic SNARE motif that comprises a stretch of approximately 60 amino acids. SNARE motifs associate into parallel four-helical bundles to form SNARE complexes. To form the synaptic SNARE complex, SNAP25 contributes two SNARE motifs, and syntaxin 1 and synaptobrevin 2 each contribute one SNARE motif. The soluble protein complexin selectively binds to the SNARE complex with high affinity and regulates fusion. Under physiological conditions, SNAREs closely cooperate with SM (SEC1/MUNC18-like) proteins and synaptic active zone proteins — MUNC13 and RAB3-interacting molecule (RIM) — to execute fusion. Studies so far suggest that these molecules either function as regulators of SNARE-dependent fusion by facilitating SNARE complex assembly or function in conjunction with SNAREs to promote fusion. In addition, the rapidity and steep Ca^{2+} dependence of the evoked synaptic vesicle fusion process occurs as a result of the interaction of SNAREs with synaptotagmin 1 and complexins. After Ca^{2+}-evoked fusion, SNARE complexes are disassembled by NSF, which is followed by recycling of used SNAREs and replenishment of vesicles from a reserve pool.

By contrast, spontaneous synaptic vesicle fusion seems to have less stringent requirements (see the figure, part **b**). For example, in addition to synaptobrevin 2, SNARE complexes nucleated by the alternative vesicular SNARE VAMP7 (vesicle-associated membrane protein 7) and VTI1A (vesicle transport through interaction with t-SNAREs homologue 1A) may selectively mediate spontaneous neurotransmission. Furthermore, these SNAREs may be involved in the formation of alternative fusion complexes with specialized functional properties that enable specific regulation of spontaneous release. Experiments have shown that increasing the number of residues that are inserted between the SNARE motif and the transmembrane region of synaptobrevin 2 in synaptobrevin 2-deficient neurons can rescue spontaneous release close to wild-type levels, but this insertion did not restore evoked release, which indicates that a suboptimal SNARE complex may be sufficient to trigger spontaneous fusion[37]. The Ca^{2+} sensitivity of spontaneous fusion seems to be mediated by C2 domain-containing proteins, such as synaptotagmin 1 and, possibly, double C2 domain (DOC2) proteins.



of these release events are carried out by the canonical vesicular SNARE synaptobrevin 2 (REF. 11). However, there is increasing evidence that spontaneous neurotransmitter release that is mediated by synaptobrevin 2 and its canonical partners SNAP25 and syntaxin 1 is differentially regulated compared with evoked release. For example, in synapses of synaptobrevin 2-deficient mice, expression of a synaptobrevin 2 construct in which 12 residues were inserted between the SNARE motif and transmembrane region was able to rescue spontaneous release, but it did not restore evoked release[37]. This suggests that the physical requirements for SNARE complex assembly are more flexible for spontaneous release. Moreover, in SNAP25-deficient neurons, expression of mutated forms of SNAP25 that resulted in destabilization of the carboxy-terminal end of the SNARE complex restored evoked release to a large extent, but it did not rescue spontaneous release. By contrast, modification of the amino-terminal end of the SNARE complex potentiated both spontaneous and evoked fusion. Importantly, both molecular manipulations had a greater impact on spontaneous release than on evoked neurotransmission[38]. In addition to synaptobrevin 2 and SNAP25, a recent report indicates that the Habc domain of syntaxin 1 is essential for spontaneous neurotransmission but that it is not required to maintain fast-evoked vesicle release[39]. Taken together, these three findings suggest that, although some spontaneous and evoked fusion events may depend on the same SNARE machinery, the molecular interactions of the same components in each case may be distinct.

These results also raise the intriguing possibility that distinct conformations of the same SNARE machinery may form a substrate for selective regulation of spontaneous versus evoked release by SNARE-interacting proteins. Consistent with this proposal, the differential requirements for spontaneous and evoked release extend to fusion machinery components other than

SNARE proteins. For example, loss of synaptotagmin 1 or synaptotagmin 2 — synaptic vesicle-associated transmembrane proteins that sense Ca^{2+} ions through their two C-terminal C2 domains — impairs fast synchronous synaptic vesicle fusion but elevates spontaneous release in a non-cell-autonomous manner[40–42]. Synaptotagmin-mutant neurons grown in mass cultures or slice cultures, but not neurons grown in isolation to form 'autapses', show increased spontaneous release[43–45]. This augmentation of spontaneous release can be suppressed by expression of Ca^{2+}-binding mutants of synaptotagmin, which suggests a key role for synaptotagmins in mediating the Ca^{2+} sensitivity of spontaneous neurotransmission[46]. At the *Drosophila melanogaster* neuromuscular junction and, in some circumstances, in mammalian neurons, loss of function of complexin — a cytoplasmic neuronal protein that binds to the SNARE complex with high affinity — results in increased spontaneous release[47,48]. These observations suggest that there may be important mechanisms that selectively suppress and regulate the probability of spontaneous release events.

Although synaptotagmins and complexins affect both evoked and spontaneous release events (albeit in opposite directions), the double C2 domain (DOC2) family of proteins selectively maintain spontaneous[49,50] or asynchronous release[51]. However, there is disagreement about whether DOC2 proteins function as Ca^{2+} sensors or as Ca^{2+}-independent regulators during spontaneous release[49,50]. Furthermore, a recent study at the *D. melanogaster* neuromuscular junction identified molecular interactions of the vacuolar ATPase V0a1 subunit in the Ca^{2+}-dependent regulation of spontaneous release[52]. The growing list of molecular players that specifically regulate spontaneous neurotransmission shows the physiological importance of this form of release and also suggests specific molecular manipulations that can be used to selectively probe spontaneous neurotransmission and elucidate its role in neuronal signalling.

Distinct vesicle pools. The molecular manipulations described above provide strong evidence for divergence between the presynaptic machineries that give rise to spontaneous versus evoked synaptic vesicle fusion. However, despite their mechanistic insight, these manipulations inevitably leave open the question of whether the processes of spontaneous and evoked neurotransmission are segregated under physiological circumstances in unperturbed synapses. The traditional assumption that the vesicles that give rise to spontaneous release and those found within the RRP share a common identity provides specific testable predictions about the relationship between the two forms of release. According to this premise, for example, the propensity for spontaneous fusion at a single release site should be directly proportional to the number of vesicles within the RRP, as each vesicle is expected to have a small but measurable propensity to fuse spontaneously. Therefore, depletion of RRP vesicles should also impair subsequent spontaneous release events, and long-term exhaustion of spontaneously recycling vesicles should reduce evoked release.

Cross-depletion experiments using manipulations to impair presynaptic function in a use-dependent manner have explicitly tested these assumptions. Initially, it was demonstrated that sustained prevention of neurotransmitter filling of spontaneously recycling vesicles using the vacuolar ATPase inhibitor folimycin impairs spontaneous release events but results in a modest reduction in subsequent evoked release[53–55]. However, under the same conditions, strong depletion of evoked release by sustained elevated K^+ stimulation impaired both evoked and spontaneous events, which indicates that large increases in presynaptic Ca^{2+} levels could mobilize all vesicles indiscriminately[56]. This observation is consistent with optical experiments that demonstrated that under strong stimulation (>20 Hz) there were no marked distinctions between the vesicle populations that recycle at rest or during activity[57,58].

However, later experiments using the small-molecule dynamin inhibitor dynasore showed that inhibition of GTP hydrolysis by dynamin does not affect spontaneous neurotransmission; instead, it causes robust depletion of vesicles within the RRP and use-dependent suppression of evoked release[59]. This finding strongly suggests that spontaneous and evoked neurotransmission originate from distinct pools of vesicles that can be distinguished by their reliance on dynamin function. This result also agrees with the finding that after genetic deletion of two major neuronal dynamin isoforms (dynamin 1 and dynamin 3), which are required for evoked synaptic vesicle endocytosis, a substantial proportion of spontaneous release is maintained[60]. However, a role for dynamin (possibly dynamin 2) in spontaneous synaptic vesicle endocytosis cannot be excluded: at the *D. melanogaster* neuromuscular junction, the temperature-sensitive dominant-negative dynamin mutant *shibire* shows use-dependent suppression of evoked and spontaneous release[61]. In contrast to the swift recovery of the RRP and evoked release after strong vesicle depletion, the recovery of spontaneous release is delayed and requires repopulation of the reserve pool, which supports the idea that spontaneous and evoked release originate from distinct vesicle pools[61]. Taken together, these results show that strong activity-dependent stimulation can indiscriminately mobilize vesicle pools, whereas moderate activity may use a distinct pool of vesicles that strictly require specific dynamin isoforms for recycling. By contrast, vesicles that normally fuse spontaneously can only respond to strong stimulation, and their recycling does not depend on the major neuronal dynamin isoforms.

The premise that spontaneous and evoked synaptic vesicle recycling pathways are segregated also received initial support from optical experiments that monitored the uptake and release of styryl dyes. In particular, experiments using the styryl dye FM2-10 showed that the kinetics of dye destaining is strictly dependent on the presence or absence of stimulation during the dye-loading phase; vesicles that were stained in the absence of activity were only mobilized to a small extent during activity[55]. These experiments also demonstrated that spontaneously endocytosed vesicles are more likely to be reused spontaneously[55]. Later experiments using the alternative styryl dye

FM1-43 provided contradictory results[56], possibly owing to the influence of prior background activity on FM1-43 uptake after neuronal silencing[56,59]. Therefore, the dye species used (FM2-10 versus FM1-43) and problems with unequivocal identification of genuine presynaptic terminals after spontaneous dye uptake were key factors that affected these results[56]. Subsequent studies revealed that multiple synaptic vesicle recycling pathways operate in parallel even under resting conditions[59].

These initial experiments motivated the recent use of molecularly specific imaging approaches to address the diversity of synaptic vesicle trafficking pathways that maintain spontaneous and evoked release[62]. For example, a biotinylated variant of synaptobrevin 2 was used to label recycling vesicles, sequentially and irreversibly, with different colours under different conditions. These experiments showed that fluorescently labelled spontaneously recycling vesicles have minimal quantitative overlap with labelled activity-dependent recycling vesicles[63]. A recent study used SGC5, a new-generation styryl dye, to examine the movement of single synaptic vesicles and revealed that the mobility of vesicles that are endocytosed after spontaneous fusion is reduced compared with vesicles that endocytose during activity[64].

Taken together, studies to date suggest that trafficking pathways that maintain spontaneous versus evoked synaptic vesicle recycling diverge. This divergence may arise from parallel trafficking of molecularly distinct synaptic vesicle populations that are, in part, marked by a non-overlapping distribution of vesicular SNARE proteins. However, it is likely that the molecular composition of these divergent populations may show partial overlap or that the vesicle composition can be dynamically altered during recycling, which results in mixing among vesicle pools. For example, the findings that a distinction between evoked and spontaneous synaptic vesicle recycling is more prominent at moderate stimulations and that strong stimulation can result in indiscriminate release are consistent with a partial overlap among vesicle pools that can be controlled by activity. Future studies that aim to biochemically characterize the various populations of synaptic vesicles, together with super-resolution imaging experiments with improved spatiotemporal resolution, will help to construct a more concrete molecular picture of synaptic vesicle heterogeneity and its functional impact on neurotransmission[62].

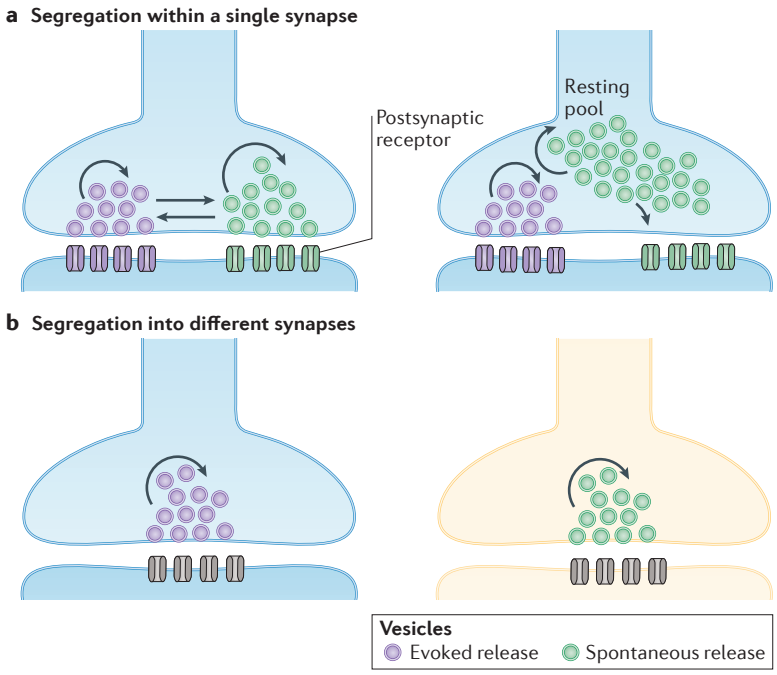


Figure 3 | **Segregation of spontaneous and evoked neurotransmission.** **a** | Studies in hippocampal synapses suggest that spontaneous and evoked fusion events may be mediated by separate pools of vesicles within the same synapse (left panel)[55,59]. According to another model (right panel), spontaneous neurotransmitter release events may originate from the resting pool of vesicles that are normally unresponsive to activity and that do not typically contribute to evoked neurotransmission[63]. In accordance with the evidence from studies of the postsynaptic region[65], these observations suggest that distinct receptor populations are activated by spontaneous or evoked neurotransmitter release in hippocampal neurons. This model is also consistent with observations of spontaneous and evoked vesicle fusion in goldfish retinal bipolar cells[69]. **b** | Alternatively, some synapses may have a strong propensity for spontaneous fusion, whereas other synapses may preferentially release neurotransmitter in response to action potentials. For example, immature synaptic boutons typically favour spontaneous release and fail to respond to action potential stimulation[104,105], which raises the possibility that a population of nascent synapses in an otherwise mature synaptic network may selectively sustain spontaneous release. In addition, some presynaptic terminals may support action potential-driven release with negligible concurrent spontaneous vesicle exocytosis.

Distinct postsynaptic targets. A complementary set of studies have investigated the potential postsynaptic counterpart to the presynaptic segregation of spontaneous and action potential-evoked synaptic vesicle recycling pathways. An early study took advantage of the use-dependent NMDA receptor antagonist MK-801 and revealed that complete blockade of spontaneous NMDA miniature excitatory postsynaptic currents (mEPSCs) results in unaffected evoked neurotransmitter release by subsequent NMDA receptor-mediated responses. The lack of interaction between the block of spontaneous NMDA mEPSCs and evoked NMDA EPSCs provides evidence that spontaneous and evoked release activate non-overlapping populations of postsynaptic NMDA receptors[65]. A later study implemented a similar strategy using philanthotoxin, which selectively blocks GluA2 subunit-deficient AMPA receptors in a use-dependent manner, to demonstrate that AMPA receptors are also segregated with respect to their responsiveness to spontaneous or evoked neurotransmission[66].

Spatial segregation. In addition to using distinct presynaptic and postsynaptic elements, it has been suggested that spontaneous and evoked neurotransmitter release occur at different locations within the synapse (FIG. 3). Direct visualization of a physical segregation of evoked and spontaneous neurotransmission processes in mammalian synapses has been difficult. Super-resolution microscopy experiments have provided evidence for the existence of nanodomains of postsynaptic receptor clusters and multiple subpopulations of signalling molecules, such as calcium/calmodulin-dependent protein kinase type II (CaMKII), which are consistent with the idea of functionally segregated neurotransmission and signalling[67,68]. In hippocampal neurons, most synapses (~70%) exhibit spontaneous

and evoked synaptic vesicle fusion, whereas the remaining synaptic terminals show a preference for spontaneous or evoked release. Using total internal reflection fluorescence microscopy, evidence for the spatial segregation of different types of neurotransmission was reported using single-vesicle imaging of goldfish bipolar cell ribbon synapses. Vesicles released as a result of stimulation were predominantly localized to the ribbon, whereas spontaneous release often occurred at extra-ribbon sites[69]. A recent study at the *D. melanogaster* neuromuscular junction used transgenic expression of the fluorescent Ca^{2+}-sensor protein GCAMP5 at the postsynaptic muscle membrane. In this setting, GCAMP5 concentrates at subsynaptic regions of muscle fibres and functions as a sensor for Ca^{2+} influx through postsynaptic glutamate receptors. Using this method, unitary spontaneous, as well as action potential-evoked, postsynaptic Ca^{2+} transients could be measured[70]. In most subsynaptic spots, the coexistence of spontaneous and evoked signals was observed. However, approximately 22% of all synaptic regions selectively participated in spontaneous neurotransmission. Importantly, in synaptic boutons that maintain both evoked and spontaneous neurotransmission, there was no significant correlation between the propensities of the two forms of neurotransmitter release. A similar study revealed substantial separation among the loci for evoked and spontaneous neurotransmission[71]. Interestingly, this study also reported a striking inverse correlation between the propensities for evoked and spontaneous fusion events (BOX 2).

Overall, these studies provide a new perspective on the relationship between evoked and spontaneous release processes at single synapses. In contrast to the classical view in which spontaneous and evoked fusion propensities at individual synapses are expected to be correlated, these studies demonstrate that the two forms of release show either no correlation[65,70] or inverse correlation[71] at certain synapses. Moreover, these findings suggest that the nature of the relationship between spontaneous and evoked release and their functional influence are likely to vary among distinct types of synapses[72]. Furthermore, it is probable that there may be developmental effects on spontaneous and evoked release. This is supported by the reciprocal regulation of immature and mature forms of synaptic vesicle recycling by the synaptic cell adhesion molecule NCAM during mammalian synapse development[73].

Selective neuromodulation of spontaneous release. Although increasing evidence highlights the segregation of spontaneous and evoked neurotransmitter release processes at synapses, the physiological relevance of this segregation has only begun to emerge recently. Experiments in multiple preparations have reported cases in which spontaneous and evoked release events are differentially regulated by neuromodulators and various cellular signal transduction pathways[21]. These include presynaptic metabotropic glutamate receptors[74], nitric oxide species[75] or other putative retrograde messengers[76], antimalarial drugs[77], induction of endoplasmic reticulum stress signalling pathways, manipulation of transcriptional mechanisms[78,79], alterations in presynaptic cholesterol homeostasis[80,81], and manipulation of presynaptic γ-secretase machinery and neuronal Ca^{2+} homeostasis[82].

This long list of observations raises the question of the potential mechanisms at work. Currently, two non-mutually exclusive mechanisms have been proposed to account for the differential regulation of spontaneous and evoked release. As indicated above, distinct fusion machineries, which are possibly nucleated by alternative SNAREs, or distinct molecular interactions of the same fusion complex may form a substrate for differential regulation. This hypothesis has received support from a recent study that demonstrated that the secreted glycoprotein reelin functions as a selective regulator of spontaneous neurotransmitter release by specifically targeting a pool of vesicles that express VAMP7 (REF. 33) (FIG. 4). Other vesicular SNAREs, such as VTI1A or the Habc domain of syntaxin 1, may be targets for other signalling pathways[27,39]. Therefore, signal transduction pathways that specifically target the SNARE machinery through post-translational modifications may elicit differential effects on spontaneous and evoked release.

Alternatively, distinct Ca^{2+} signalling pathways that are relatively spatially isolated may differentially regulate the two forms of release. This hypothesis has received support from work that investigated glutamatergic transmission from solitary tract afferents, which found that activation of presynaptic transient receptor potential cation channel subfamily V member 1 (TRPV1) selectively regulated asynchronous or spontaneous release events[83]. The same group of researchers also showed that evoked responses, but not spontaneous release events, could be swiftly inhibited by activation of presynaptic cannabinoid 1 receptors, whereas inhibition of TRPV1 selectively suppressed spontaneous release[84]. These results show two neuromodulatory pathways that coexist at individual nerve terminals and that can independently modulate spontaneous and evoked vesicle fusion events at the same terminal.

Recent studies also provide evidence that, in central synapses, voltage-gated Ca^{2+} channels are important physiological triggers for spontaneous release events because

Total internal reflection fluorescence microscopy
A high-resolution fluorescence microscopy technique that takes advantage of a laser-induced evanescent wave of fluorescence emission which is very close to the interface of two media that have different refractive indices.

Ribbon synapses
Synapses characterized by an electron-dense ribbon or bar in the presynaptic terminal. The ribbon is commonly oriented at a right angle to the membrane and sits just above an evaginated ridge. It is thought that the ribbons help to guide vesicles to the release sites. Ribbon synapses are commonly found in the retina and cochlea of vertebrates.

Box 2 | **Propensities for spontaneous and evoked fusion within a synapse**

According to the classical perspective, spontaneous neurotransmitter release events originate from the same pool of vesicles that are docked and primed for fusion (that is, the readily releasable pool). According to this model, at a single release site, the probability of evoked vesicle fusion is expected to show strong correlation with the propensity for spontaneous release. However, earlier experiments in hippocampal synapses[65] and a recent study at the *Drosophila melanogaster* neuromuscular junction[70] have challenged this view and showed that, at an individual release site, spontaneous and evoked release events do not show significant correlation. Another study that was conducted at the *D. melanogaster* neuromuscular junction showed substantial separation among the loci for spontaneous and evoked neurotransmission[71]. However, these experiments revealed an inverse correlation between the propensities for evoked and spontaneous fusion events. Although accumulating evidence counters the classical perspective and supports the assumption that spontaneous and evoked fusion events are, to a large extent, independent within individual synapses, these recent studies also raise new questions on the exact nature of the relationship between the two release pathways.

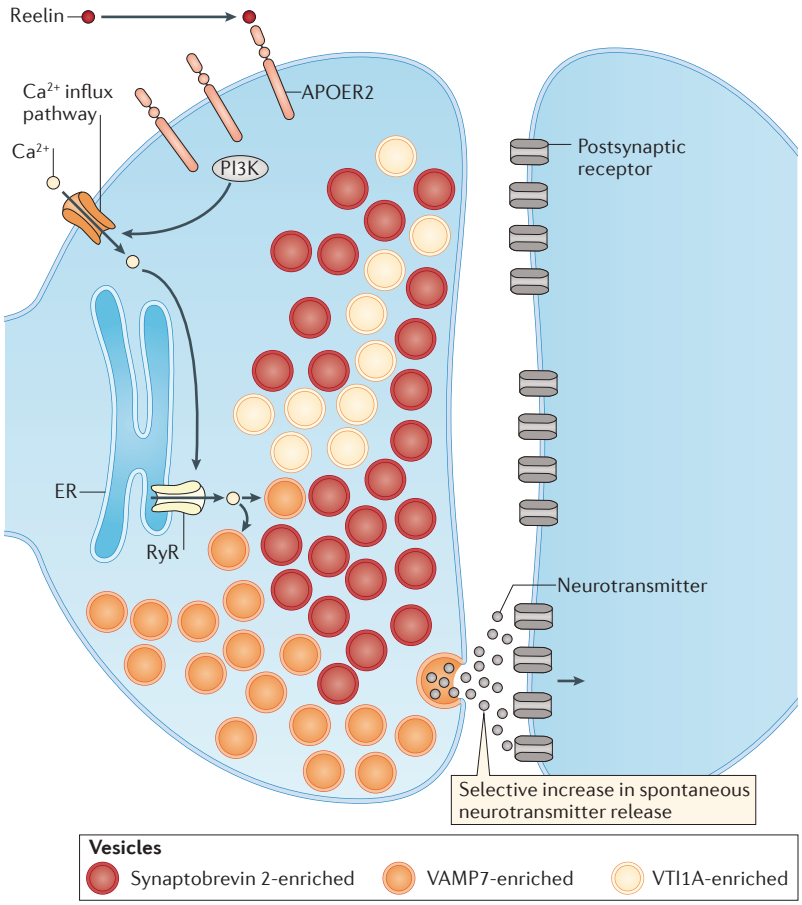


Figure 4 | **An emerging model of the distributions of vesicular proteins among synaptic vesicle pools.** The model shown suggests that spontaneous and evoked fusion may occur in the same synapses but be carried out by separate pools of vesicles that may recycle independently. Moreover, this model suggests that vesicles that recycle spontaneously may have an intrinsic molecular difference that renders them selectively vulnerable to certain signal transduction pathways. At central synapses, synaptobrevin 2 is the predominant vesicular SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) protein that ensures rapid execution of synaptic vesicle fusion. However, studies of synaptobrevin 2 suggest that a parallel pathway involving non-canonical SNAREs may mediate fusion and recycling of a subset of vesicles[10,11]. Work has revealed that both VTI1A (vesicle transport through interaction with t-SNAREs homologue 1A) and VAMP7 (vesicle-associated membrane protein 7) could fulfil this role and specifically traffic at rest. Molecularly distinct synaptic vesicle populations with different fusion properties enable regulatory pathways to selectively influence a particular type of neurotransmission, thereby triggering a specific cellular response. In this way, the nature of presynaptic activity can determine the influence of downstream postsynaptic signalling events. This model can account for the selective regulation of spontaneous neurotransmission by reelin. According to this model, reelin mediates activation of APOER2 (also known as LRP8) and downstream Ca^{2+} signalling, which leads to the selective mobilization of VAMP7-enriched synaptic vesicles. In this scenario, VAMP7-dependent fusion machinery may be spatially distant from other vesicle pools and may also be associated with a Ca^{2+}-sensing protein that is tuned to changes in resting-level Ca^{2+} signals. The identity of this protein remains to be identified[33]. ER, endoplasmic reticulum; PI3K, phosphatidylinositol 3-kinase; RyR, ryanodine receptor.

these channels open stochastically at rest[85–87]. It has been proposed that among the presynaptic P-, Q-, N- and R-type Ca^{2+} channels, the relatively low-voltage activation of R-type channels probably have a prominent role in spontaneous neurotransmitter release[87]. As presynaptic voltage-gated Ca^{2+} channels form a crucial substrate for neuromodulation, selective regulation of these channels by neuromodulators may also confer differential sensitivity to spontaneous release events.

Despite the increasing evidence for a potential role for differential Ca^{2+} signalling pathways in regulation of spontaneous versus evoked release, the exact nature of the Ca^{2+} dependence of spontaneous release remains an open question; for example, striking differences between excitatory and inhibitory synapses, in terms of their dependence on spontaneous voltage-gated Ca^{2+} channel openings, have been reported[85,88]. In the calyx of Held, loss of synaptotagmin 2 resulted in an increase in mEPSC frequency that was sensitive to application of the fast exogenous Ca^{2+} buffer BAPTA, but not to application of the slower buffer EGTA or extracellular Ca^{2+} ions, which suggests a role for rapid Ca^{2+} transients that originate from internal stores[89]. By contrast, in wild-type calyces, mEPSCs did not show sensitivity to BAPTA, a finding that is consistent with earlier observations in hippocampal synapses[89,90]. Interestingly, in wild-type hippocampal synapses, prolonged incubation with a membrane-permeable BAPTA variant can also suppress a large proportion of spontaneous release[46]. Taken together, these findings support the role of Ca^{2+} ions as a crucial regulator of the rate of spontaneous neurotransmitter release. However, the nature of this Ca^{2+} sensitivity seems to be highly dependent on synapse type and is probably influenced by non-cell-autonomous factors[41,42].

Functions of spontaneous release

Postsynaptic signal transduction cascades. The rate of spontaneous neurotransmission per synaptic bouton is estimated to be around 0.01 Hz[43,55,91]. However, certain neuromodulators, such as acetylcholine, may elicit an approximately 50-fold increase in the rate of spontaneous release events at individual synapses, which in turn facilitates electrotonic summation of events at the dendritic level[17]. In this way, spontaneous release events influence electrical activity and have a global impact on neuronal excitability. In addition, single quantal release events may regulate action potential firing and subsequent neuronal signalling in compact neurons with high membrane resistance[92]. This electrical influence of spontaneous release events may indirectly trigger downstream neuronal signalling. In addition, experiments that were conducted after suppression of neuronal activity indicate that local mechanisms that are activated by individual spontaneous release events at low frequency can also directly elicit Ca^{2+} signals at rest. Postsynaptic metabotropic glutamate receptors (particularly mGluR1 and mGluR5) or Ca^{2+}-permeable AMPA receptors that lack GluA2 subunits are possible transducers for these spontaneous Ca^{2+} signals; in addition, there is strong evidence that single glutamate release events can activate NMDA receptors at rest[53,93–97]. This characteristic of NMDA receptors arises because there is an incomplete Mg^{2+} block at resting membrane potentials[98]. Furthermore, this form of signalling can be augmented when certain NMDA receptor subunits with lower Mg^{2+} affinity (such as NR2C or NR2D) are involved[95].

Synaptic scaling
Upscaling or downscaling of the quantal amplitude of all synapses onto a postsynaptic neuron in response to long-lasting changes in neuronal activity.

Recently, a striking example of neuronal signalling mediated by activation of resting NMDA receptors has emerged from studies that showed that blockade of spontaneous NMDA receptor-driven synaptic events, but not evoked neurotransmitter release, deactivates eukaryotic elongation factor 2 (eEF2) kinase, which results in reduced eEF2 phosphorylation and reverses the suppression of dendritic protein translation[93] (FIG. 5). Later studies demonstrated that the same mechanism has a key role in mediating the rapid antidepressant action of the NMDA receptor blocker ketamine *in vivo*[53,96,97]. Moreover, the effect of NMDA receptor blockers on synaptic plasticity can be mimicked by acute selective suppression of spontaneous release by the application of a vacuolar ATPase blocker at rest, which depletes neurotransmitter from spontaneously recycling vesicles[53]. Collectively, these results suggest that selective presynaptic impairment of spontaneous release, without alterations in evoked neurotransmission, is sufficient to cause postsynaptic signalling. However, it remains unclear whether there is a direct correlation between particular forms of neurotransmitter release driven by molecularly distinct synaptic vesicle populations and specific postsynaptic responses.

In addition to regulation of eEF2 kinase signalling, studies have shown that, under resting conditions, blocking Ca^{2+} entry or direct reduction of intracellular Ca^{2+} levels using exogenous buffers induces retinoic acid synthesis and synaptic scaling (FIG. 5). These findings suggest that the removal of Ca^{2+} ions disinhibits retinoic acid synthesis, which in turn provides the stimulus for synaptic scaling[99,100]. Interestingly, the application of tetrodotoxin (TTX) to block action potentials alone does not induce retinoic acid synthesis but does produce synaptic scaling over a longer timescale. These results indicate that the Ca^{2+} influx that is associated with spontaneous rather than evoked transmission is the key signal for retinoic acid synthesis. It was also postulated that any treatment that lowers intracellular Ca^{2+} levels below those produced by resting miniature neurotransmission will induce retinoic acid synthesis. This idea is consistent with a recent study that demonstrated that altering resting Ca^{2+} signals in neurons could also change gene transcription in the nucleus[101]. These results, along with recent studies showing that resting release suppresses presynaptic function through brain-derived neurotrophic factor (BDNF) synthesis[102,103], provide support to the premise that Ca^{2+} signalling driven by spontaneous neurotransmitter release is a key regulator of homeostatic plasticity.

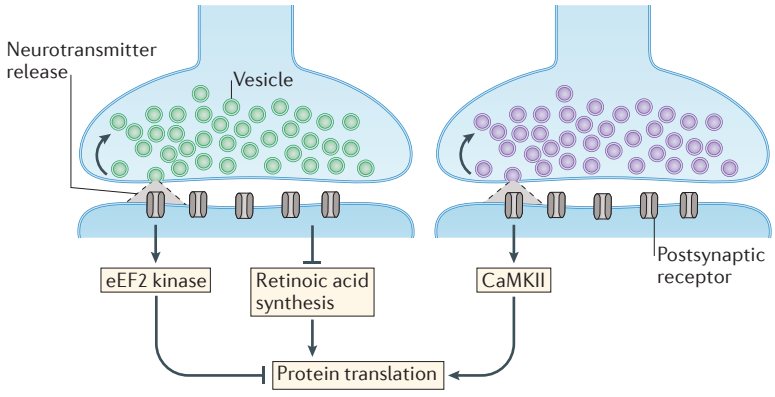


Figure 5 | **Postsynaptic signalling pathways that are differentially activated by spontaneous and evoked neurotransmitter release.** Spontaneous NMDA receptor-driven synaptic events, but not evoked neurotransmitter release, have been shown to activate eukaryotic elongation factor 2 (eEF2) kinase, which results in increased eEF2 phosphorylation and the suppression of dendritic protein translation[53,93,94]. In addition, studies have shown that, under resting conditions, suppression of dendritic Ca^{2+} signalling induces retinoic acid synthesis and synaptic scaling. These results indicate that the Ca^{2+} influx that is associated with spontaneous rather than evoked transmission is a crucial signal for suppression of retinoic acid synthesis[99,100]. By contrast, activation of dendritic calcium/calmodulin-dependent kinase II (CaMKII) requires strong activity or coincident dendritic depolarization and Ca^{2+} influx. Therefore, this pathway is preferentially activated during evoked neurotransmission[110].

Developmental importance. Spontaneous neurotransmitter release is a dominant feature of nascent synaptic contacts[104,105], and the propensity for spontaneous release events shows a gradual increase during synapse development[106]. Early experiments showed that suppression of postsynaptic receptor activation, rather than sole inhibition of neuronal activity, is required to cause developmental structural plasticity[107,108]. Furthermore, at the *D. melanogaster* neuromuscular junction, complexin-null mutants showed a marked increase in spontaneous fusion and a profound overgrowth of synapses[47]. A recent study used several loss-of-function and gain-of-function manipulations to alter spontaneous release and demonstrated that spontaneous neurotransmission, but not evoked neurotransmission, is required for the normal structural maturation of *D. melanogaster* glutamatergic synapses. This study also showed that spontaneous neurotransmission functions by using the guanine nucleotide exchange factor Trio and the Rac1 GTPase molecular signalling pathway to affect synaptic growth[109]. Although a developmental role for spontaneous release events in guiding synapse maturation is supported by these studies, it remains unclear whether the same mechanisms and signal transduction pathways discussed above also underlie the role of spontaneous release events in these developmental processes.

Role in mature networks. Most evidence so far suggests a homeostatic role for spontaneous neurotransmitter release in mammalian systems: suppression of spontaneous release events decreases postsynaptic Ca^{2+} signalling and, in turn, activates signal transduction pathways that lead to an increase in synaptic efficacy[20,110]. A similar role for resting release in homeostatic synaptic plasticity was documented at the *D. melanogaster* neuromuscular junction[111]. As indicated above, blockade of spontaneous NMDA receptor-mediated synaptic events, but not evoked neurotransmitter release, results in reduced eEF2 phosphorylation and desuppression of dendritic protein translation[93]. Recent work indicates that the same mechanism has a key role in mediating the rapid antidepressant action of the NMDA receptor blocker ketamine *in vivo*[96]. These findings raise the possibility that selective manipulation of spontaneous neurotransmitter release in mature synaptic networks may be used as a specific therapeutic strategy against neuropsychiatric disorders[112].

In addition to its role in homeostatic synaptic plasticity, spontaneous release events may also have a more instructive role in mediating plasticity, particularly under circumstances in which the rate of spontaneous release can be augmented in response to the action of neuromodulators[17,113–115]. Alternatively, spontaneous release events may have a permissive role in synaptic plasticity by sensitizing signal transduction elements to activity-dependent signalling[113]. Recent studies have also identified unconventional ways in which spontaneous release may contribute to neuronal excitability; for example, a new class of small mEPSCs ('preminis') that arise from retrograde autocrine activation of axonal receptors following spontaneous vesicle release have been characterized[116]. Although the functional importance of these preminis remains unclear, they provide a clear demonstration that spontaneous release events can influence the neurons from which they originate, in addition to their canonical postsynaptic effects.

Conclusion

This Review of recent literature on spontaneous release events highlights the growing consensus that release of a particular neurotransmitter may convey different messages to a postsynaptic neuron depending on whether it is released spontaneously or in response to presynaptic action potentials. This differential postsynaptic signalling is enabled by presynaptic segregation of vesicle trafficking pathways that mediate spontaneous and synchronous-evoked release. In some cases, this segregation may also extend to mechanisms and signalling targets of asynchronous release. Parallel signalling by kinetically diverse release processes may enable the neurotrophic, homeostatic or other functions of released neurotransmitters to be isolated from their crucial role in precise presynaptic action potential-driven information transfer. Future experiments are needed to fully determine the presynaptic mechanisms that specifically affect spontaneous release events, which will in turn aid the development of strategies to selectively manipulate spontaneous release. Moreover, it will be important to devise monitoring strategies to directly quantify spontaneous release in a pathway-specific manner in long-term *in vivo* studies of intact neuronal circuits. New strategies that selectively target spontaneous release events will provide insights into its autonomous function and address whether spontaneous release events can signal independently during ongoing activity. In addition, these studies will help us to better understand the signalling mechanisms that operate during neuronal silence and to uncover novel mechanisms that are activated following neuronal or synaptic silencing. This information will be essential to provide new insights into the synaptic processes that are affected by neuropsychiatric and neurological disorders[112].

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Competing interests statement
The author declares no competing interests.

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