The accepted theory of vesicular release of neurotransmitter posits that only a single vesicle per synapse can fuse with the membrane following action potential invasion, and this exocytotic event is limited to the ultrastructurally defined presynaptic active zone. Neither of these dictums is universally true. At certain synapses, more than a single vesicle can be released per action potential, and there is growing evidence that neuronal exocytosis can occur from sites that are unremarkable in electron micrographs. The first discrepancy extends the dynamic range of synapses, whereas the second enables faster and more robust chemical transmission at sites distant from morphologically defined synapses. Taken together, these attributes expand the capabilities of cellular communication in the nervous system.

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Introduction
Traditionally, it has been assumed that excitation of a presynaptic neuron leads to the fusion of, at most, a single synaptic vesicle with the plasma membrane at the active zone. In this manner, transmitter is released into the synaptic cleft and activates the receptors clustered just across the cleft in the postsynaptic density (PSD). Several recent studies have questioned whether vesicular release is so stereotyped.

Although several papers from the 1990s reported that evoked release at individual synapses was not restricted to single vesicles [1–3], there has been a recent avalanche of papers describing multivesicular release (MVR) at a variety of synapses, especially when the release probability is high [4–13]. However, there is good evidence that at other synapses vesicular release is limited to single vesicles no matter how high the release probability [14–16]. This univesicular constraint requires either that there is signaling among all vesicles that are capable of immediate release or that this vesicular pool comprises a single vesicle. It is not known which of these mechanisms is used or how it is over-ridden at synapses where MVR occurs.

In addition to the heresy of MVR, several reports suggest that presynaptic exocytosis is not limited to the active zone. Previously, the activation of extrasynaptic receptors and receptors at neighboring, otherwise quiescent, synapses was thought to be caused by diffusion, or spillover, of transmitter from active synapses [17,18]. In addition to spillover, however, it has been shown recently that exocytosis is not restricted to the active zone [19,20**,**21*,**22**]. Therefore, this ectopic release [23,24] could be at least partially responsible for activation of extrasynaptic receptors and synaptic receptors at non-releasing synapses (Figure 1).

Ectopic release of large, dense core vesicles versus small, synaptic vesicles
Vesicular release from neuronal membranes lacking morphological specializations is not a new finding. Large, dense-core vesicles are released from non-specialized membranes in Retzius cells [25] and isolated terminals from trigeminal [26] and hippocampal neurons [27]. However, these reports found that release of small, synaptic vesicles was restricted to active zones. Evidence for ectopic — that is, not from the active zone — release of small, synaptic vesicles first came from freeze-fracture studies of the neuromuscular junction. Following repetitive presynaptic stimulation, dimples in the membrane corresponding to fused vesicles were mostly found near the active zone but were also found more than 100 nanometers away (Figure 2a) [23]. Exocytosis evoked by increasing the potassium levels resulted in an almost uniform pattern of dimples along the presynaptic membrane that was not related to the position of the active zone [23,24]. Although these dimples could represent sites of either exocytosis or endocytosis, the authors suggested that these stimuli activate latent sites of exocytosis that might be specified by the large intramembrane particles located between active zones.

Release from retinal bipolar cell terminals
The most direct visual observation of ectopic release of small, synaptic vesicles comes from evanescent-wave microscopy of isolated retinal bipolar cell terminals [28,29]. Using this technique, stimulated exocytosis of vesicles loaded with the fluorescent dye, FM1-43, could be observed in the presynaptic membrane. Most of these
fusion events (64%) were clustered at sites that corresponded to locations of \(\text{Ca}^{2+}\) influx and, thus, putative active zones. However, the remaining fusion events were randomly dispersed in the presynaptic membrane, indicating that exocytosis was not restricted to active zones (Figure 2b). A sizable fraction of fusion events in this preparation, then, might represent ectopic exocytosis.

**Ectopic release in the cerebellum**

In the cerebellar cortex, glutamate-releasing climbing fibers (CFs) innervate Purkinje cells (PCs). These synapses are encased in the processes of Bergmann glial cells (BGs) [30], the astrocyte of the cerebellar molecular layer. Unlike most astrocytes, BGs express calcium-permeable AMPA receptors (AMPARs) that are activated following CF stimulation [31]. However, simultaneous recordings from PCs and BGs that received input from the same CF revealed that quantal events in one cell are not coincident with those in the other cell (Figure 3a and b) [19]. The absence of coincidence suggests that the two cell types monitor release from different release sites: exocytosis from active zones, in the case of PCs, and exocytosis from ectopic sites, in the case of BGs. In addition, this lack of concurrence indicates that spillover of glutamate from either site is not sufficient to activate AMPARs associated with the other site, that is, low affinity receptors such as AMPARs require nearby release. The glutamate concentration transient responsible for BG quanta was estimated to reach a peak concentration of 1.5 millimolar (mM) [21*], a level similar to those reported at synapses [12,32]. Although distinct presynaptic membrane specializations were not found adjoining BG processes, vesicles were observed next to presynaptic membranes apposing BG processes (Figure 3c) [21*], providing a possible supply for ectopic exocytosis.

Consistent with two distinct sites of release, evoked responses recorded in PCs and BGs are affected differently by manipulations that alter release. CF to BG
AMPAR responses are more sensitive to application of the membrane permeable Ca\(^{2+}\) chelator, EGTA-AM, which limits presynaptic Ca\(^{2+}\) transients to the vicinity of Ca\(^{2+}\) channels, than CF to PC excitatory postsynaptic currents (EPSCs) are [19]. Similarly, CF to BG AMPAR currents are inhibited to a much greater degree by the N-type Ca\(^{2+}\) channel blocker, \(\omega\)-conotoxin GVIA, than CF to PC EPSCs are [20]. These results indicated that at CFs, exocytosis from active zones and ectopic sites is differentially coupled to Ca\(^{2+}\) influx.

Quantal events were evoked in BGs not only by stimulation of CFs but also by stimulation of parallel fibers (PFs) [19], the other source of excitatory input to PCs. Quantal events represent the synchronous binding and activation of a number of ligand-gated ion channels, a feat that requires the exposure of these receptors to rapidly rising, high concentration transients of transmitter. Because PF-evoked quantal events had similar kinetic properties to those from CFs, it is likely that ectopic release provides fast and direct input to BGs from both the high release probability CF varicosities and the more conventional low release probability, en passant PF boutons.

**Ectopic release in the ciliary ganglion**

At cholinergic calyceal synapses in the chick ciliary ganglion, two types of neuronal nicotinic acetylcholine receptors (nAChRs) have non-overlapping distributions. The \(\alpha_3\)-nACh subunit-containing receptors are located at synapses and at extrasynaptic sites, whereas the \(\alpha_7\)-nAChRs are mainly excluded from synapses [33–36]. However, \(\alpha_7\)-nAChRs, not \(\alpha_3\)-nAChRs, largely mediate evoked EPSCs at this synapse [37,38]. Ultrastructural markers of releasable vesicles are found throughout the presynaptic calyx, not just apposing the PSD [36]. If vesicles docked at presynaptic sites apposing \(\alpha_7\)-nAChRs are capable of evoked release, they could mediate the bulk of the EPSCs. Coggan et al. [22] tested this hypothesis by combining computer simulations of transmitter diffusion and receptor kinetics with electron microscopic reconstruction of a portion of a calyceal synapse that included active zones and adjacent
extrasynaptic domains. Simulated synaptic activity reproduced experimental recordings of miniature excitatory postsynaptic currents only when ectopic transmission was included in the model. This study suggests that ectopic release of neurotransmitter can participate in interneuronal synaptic transmission in addition to in neuronal–glial communication [19].

**Dendritic ectopic release**

A number of studies indicate that vesicular release can occur from neuronal dendrites without the assistance of active zones. Dendritic release of both GABA (γ-aminobutyric acid) and glutamate elicited by back-propagating action potentials has been reported to regulate both excitatory and inhibitory axo–dendritic synapses between neocortical pyramidal cells and interneurons [39]. These release events are blocked by intracellular BAATPA, a Ca\(^{2+}\) chelator, and botulinum toxin D, and are thus likely to be mediated by exocytosis. Similarly, GABAergic responses evoked among the dendrites of olfactory bulb periglomerular neurons are dependent on extracellular Ca\(^{2+}\) and sometimes have kinetics similar to those of mIPSCs, suggesting direct exocytosis from one dendrite onto its neighbor [40]. Dopaminergic neurons of the substantia nigra release dopamine from their dendrites. As measured by carbon fiber amperometry, dopamine release from these dendrites is rapid and Ca\(^{2+}\)-dependent, which are both hallmarks of vesicular release [41]. In none of these cases is there solid evidence that the dendritic release occurs from conventional active zones, suggesting that ectopic release is not the sole privilege of axonal structures.

**Exocytosis from hair cells**

Using electron tomography of frog saccular hair cells, Lenzi et al. [42] found that vesicles not only cluster around active zone-associated synaptic ribbons (or synaptic bodies) but also populate remote sites as solitary ‘outliers’. Vesicles at both locations were depleted in approximately equal proportions by depolarization. In addition, Beutner et al. [43] found that Ca\(^{2+}\) uncaging in mouse inner hair cells (IHCs) increased the electrical capacitance of the membrane of the cell by a value that would correspond to the fusion of ~40,000 vesicles. This number of fusion competent vesicles greatly outnumber the morphologically defined docked vesicles at the active zones of IHCs (~300) [44]. The authors conclude that the exocytosis of fusion-competent vesicles at some distance from the active zones contributes to the slower, sustained phase of release during prolonged stimulation (but see Edmonds et al. [45] for discussion of compound fusion). Recently, Khimich et al. [46] found that in mice expressing a mutant form of Bassoon, the presynaptic scaffolding protein, ribbons were not associated with the active zones in IHCs. Even though the readily releasable pool of vesicles was reduced in these IHCs and the rapid, synchronous phase of release was inhibited, they were still capable of sustained exocytosis. This observation supports the idea that the ribbon is required for synchronous release of vesicles, but that additional fusion outside the active zone could occur without help from the ribbons.

**Mechanism of ectopic release**

Fusion of synaptic vesicles at the presynaptic active zone requires the SNARE (soluble NSF attachment receptor) core complex that comprises three proteins: the two target-SNAREs, SNAP-25 (synaptosome-associated protein 25 kDa) and syntaxin 1, and the vesicle-SNARE, synaptobrevin [47]. Both SNAP-25 and syntaxin 1 are expressed throughout the axolemma, even in membranes surrounded by myelin [48,49], whereas other active zone proteins are concentrated at the active zone at 100-fold higher densities than they are in surrounding membranes [49]. It remains to be determined which subset of proteins that participate in the cycling of the SNARE complex at the active zone is also present at ectopic sites.

Synaptic vesicles docked far from the active zone are farther from the main site of Ca\(^{2+}\) entry than those at the active zone. However, the plasma membrane outside active zones is not completely devoid of Ca\(^{2+}\) channels [50]. In this regard, CF release onto BGs is more sensitive to divalent cation Ca\(^{2+}\) channel blockers than CF release onto PCs is [51]; it might be that the ectopic release machinery is more tightly associated with the sparse, ectopic Ca\(^{2+}\) channels than it is with Ca\(^{2+}\) channels at the active zone. The N-type Ca\(^{2+}\) channel blocker, ω-conotoxin GVIA, blocks the BG AMPAR response much more than it blocks the PC EPSC, indicating that not only coupling of Ca\(^{2+}\) influx to release is different but that a different set of Ca\(^{2+}\) channels is required for ectopic release [20**]. However, repeated stimulation of PFs results in greater facilitation of BG AMPAR responses than that of PC EPSCs. This suggests that presynaptic Ca\(^{2+}\) accumulation is larger and/or more effective at ectopic sites. Finally, it appears that the ectopic release is more sensitive to modulators of release than synaptic release is [51,52]. Although the requirements for release at ectopic sites are not as well defined as those for synaptic release, it is clear that there are a number of structural and mechanistic differences between the two.

**Consequences of ectopic release**

Release of transmitter at ectopic sites will activate extrasynaptic receptors, a phenomenon previously attributed exclusively to spillover from the synaptic cleft. Modeling studies [22**,-53] suggest that high concentrations of transmitter are short-lived and restricted to locations very near the site of release. Indeed, diffusion of glutamate from CF to PC synapses following univesicular release appears to be insufficient to activate AMPARs in the surrounding BG membranes [19]. Thus, receptors that require high glutamate concentrations for activation cannot be located more than a few hundred nanometers from...
the origin of release. In regions where release sites are densely packed, spillover and pooling of glutamate from releasing synapses can activate receptors in neighboring, non-releasing synaptic clefts [54]. Even in these structures, ectopic release could contribute to spillover. A number of experiments suggest that spillover, even following multivesicular release [7,12], normally mediates only a small portion of the CF-stimulated BG AMPAR response [19,20**,21*], indicating that normally, low affinity extrasynaptic receptors are poorly activated by spillover alone. The high affinity BG glutamate transporters, however, are mainly activated by spillover from the synaptic cleft, indicating that glutamate can diffuse for significant distances, albeit at low concentrations [20**]. Given that these transporters are, on average, closer to ectopic release sites than to active zones, the great majority of glutamate must be released within the cleft. The major advantage of ectopic release appears to be the production of high concentration transients of transmitter outside of the synaptic cleft.

Physiological role of ectopic release

What is the physiological role of ectopic release in the cerebellum? Ectopic release is required to activate a significant AMPAR conductance in BGs. Ca2+ influx through these AMPARs appears to be required for maintaining the encasement of excitatory synapses on PCs by BG processes [55]. Rendering the AMPAR conductance Ca2+-impermeable by over-expression of the edited GluR2 subunit causes a dramatic retraction of fine BG processes and a prolongation of the time required for glutamate clearance [55]. The envelopment of synapses by BG processes ensures that there is a high density of glial transporters close to sites of release, which, along with the physical encasement, helps to isolate neighboring synapses from one another.

At calyceal synapses in ciliary ganglia, ectopic release of acetylcholine might be the predominant mechanism of activation of extrasynaptic α7-nAChRs [22**]. Rapid and efficient activation of α7-nAChRs is necessary to sustain high frequency throughput. Thus, ectopic release is necessary for the proper functioning of this ganglion as diffusion of ACh from neighboring synapses is inadequate for robust α7-nAChR activation.

Conclusions

Although ectopic release has been revealed in several structures, whether it occurs throughout the nervous system is not known. This is at least partly because sensors that exclusively report ectopic release are not expressed in most parts of the nervous system; BG AMPARs are relatively unique in this regard. If ectopic release from pre- and possibly post-synaptic elements is the rule, it will be necessary not only to distinguish its effects from those of synaptic release but also to understand its regulation.
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20. Matsui K, Jahr CE: Differential control of synaptic and ectopic vesicular release of glutamate. *Neurosci* 2004, 24:8932-8939. The authors studied a number of attributes of climbing and parallel fiber responses in Purkinje cells and Bergmann glial cells to determine if two different release processes were responsible for activating AMPA receptors. Paired-pulse modulation was different in the two cell types, suggesting different release mechanisms. In addition, selective block of N-type Ca2⁺ was much more effective at blocking climbing fiber AMPA receptor responses in Bergmann glial cells than it was in Purkinje cells. Interestingly, climbing fiber-stimulated glutamate transporter currents in Bergmann glial cells behaved more similarly to Purkinje cell EPSCs than in Bergmann glial AMPA receptor responses, both in their response to paired stimulation and in their susceptibility to N-type Ca2⁺ block. This suggests that the high affinity glutamate transporters are better at sensing lower concentrations of glutamate subsequent to spillover than the low affinity AMPA receptors. This also indicates that synapses, not ectopic sites, are the source of the majority of glutamate released by exocytosis.

21. Matsui K, Jahr CE, Rubio ME: High-concentration rapid transients of glutamate mediate neural-glial communication via ectopic release. *J Neurosci* 2005, 25:7538-7547. By combining immunogold electron microscopy with electrophysiology, the authors determined the density of AMPA receptors expressed by Bergmann glial membranes that surround excitatory synapses on Purkinje cells. Using a kinetic model of Bergmann glial AMPA receptors, they estimated that the glutamate concentration transient to which these AMPA receptors are exposed reaches a peak concentration of about 1.5 mM, which decays with a time constant of about 0.5 ms. In addition, they found electron microscopic evidence for synaptic vesicles located at the cytoplasmic face of parallel fiber and climbing fiber presynaptic elements that were directly apposed to Bergmann glial membranes, indicating that this could be a vesicular pool responsible for ectopic release.

22. Coggan JS, Bartol TM, Esquenazi E, Stiles JR, Lamont S, Martone ME, Berg DK, Ellisman MH, Sejnowski TJ: Evidence for ectopic neurotransmission at a neuronal synapse. *Science* 2005, 309:446-451. The authors used computer simulations with electron microscopic tomography to determine that ectopic release is required for normal transmission in the ciliary ganglion. The results of these simulations provide an elegant solution to the long-standing puzzle that α7-nAChRs are activated by presynaptic release but are not apposed to ultrastructurally defined active zones.


The authors report that the anchoring of synaptic ribbons to presynaptic active zones in inner hair cells is disrupted in mice expressing mutant Bassoon, a scaffolding protein. The physiological consequence of this is a decrease in the readily releasable vesicle pool and a disruption of signaling in postsynaptic spiral ganglion neurons. These cells were still capable of sustained exocytosis, indicating that ribbons are not required for this component of release.


