Neurotransmitter: Release

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Advanced Article

Article Contents

The nervous system is composed of networks of cells that engage in coordinated circuits to permit neural function. Within these precise neural circuits, communication between individual cells is primarily chemical in nature. Neurotransmitter release via exocytosis of neurotransmitter-filled synaptic vesicles is a fundamental step in this process. Here we overview the biochemical processes that regulate exocytotic neurotransmitter release by focusing on three key stages: 1) loading of neurotransmitter into synaptic vesicles, 2) synaptic vesicle docking and priming reactions, and 3) calcium triggering of the vesicle fusion reaction. We also introduce the controversial topic of fusion-pore modulation as it pertains to the release of neurotransmitter. Lastly, we discuss current methods for detecting and quantifying neurotransmitter release.

Biological Relevance of Neurotransmitter Release

The process of information flow between neurons is termed synaptic transmission, and in its most basic form it is characterized by unidirectional communication from the presynaptic to postsynaptic neuron. The process begins with the initiation of an electrical impulse in the axon of the presynaptic neuron. This electrical signal—the action potential—propagates to the axon terminal, which thereby stimulates the fusion of a transmitter-filled synaptic vesicle with the presynaptic terminal membrane. The process of synaptic vesicle fusion is highly regulated and involves numerous biochemical reactions; it culminates in the release of chemical neurotransmitter into the synaptic cleft. The released neurotransmitter diffuses across the cleft and binds to and activates receptors on the postsynaptic site, which thereby completes the process of synaptic transmission.

Neurotransmitter release is not assured in response to synaptic stimulation. Rather, the process of vesicle fusion for individual release-competent vesicles is probabilistic. This process confers a discrete probability (between 0 and 1) that a given synapse will release neurotransmitter after an action potential (the *synaptic* release probability). For the majority of synapses in the central nervous system, the release probability at a defined synaptic contact is below 0.3, which leads to the often-quoted statement that the release process is "reliably unreliable" (1). Despite this fact, it has been demonstrated that some central nervous system synapses (in a variety of brain regions) do exhibit release probabilities as high as 0.9 (2–4). This higher *synaptic* release probability could be explained by more release-competent vesicles and/or because the *vesicular* release probability of the individual release-competent vesicles is higher. Moreover, release probability is highly dynamic; it incorporates several forms of short-term plasticity (5).

Although this article focuses almost exclusively on the essential aspects of release of classic small-molecule neurotransmitters from neurons of the central nervous system, it is appropriate to mention that the neurotransmitter release process encompasses several additional salient facets. Release of neurotransmitters from neurons can occur at various locations on the neuron (e.g., axo-dendritic, axo-somatic, axo-axonal, dendro-dendritic, and, in the case of the neuromuscular junction, from axon to muscle). In addition, neurotransmitter release can occur from various different cell types (e.g., neuroendocrine cells and glial cells), in which case it can be debated whether the use of the term neurotransmission is truly appropriate. Release of neuropeptides is typically from dense-core vesicles rather than small synaptic vesicles, but many mechanisms parallel those for classic neurotransmitters. A group of diffusible messengers that includes nitric oxides, endocannabinoids, and hydrogen peroxide is often classified as retrograde neurotransmitters. However, these messengers are synthesized *de novo* rather than stored in vesicles and released, and thus they will not be considered here. Finally, even in the case of release of classic neurotransmitters from neurons of the central nervous system, highly specialized synapses have been described and investigated in great detail (e.g., ribbon-type synapses of the retina and Calyx-type synapses in the auditory pathway). These collective special considerations, although not adequately discussed herein, serve as excellent examples of the wide diversity

of signaling mechanisms employed by the nervous system to achieve information transfer.

Biological Chemistry of Neurotransmitter Release

Within the presynaptic terminal, neurotransmitter-filled vesicles are clustered tightly in high numbers. The first electron micrograph images of synapses in which clusters of synaptic vesicles could be seen clearly were obtained in the mid-1950s (6, 7). This work coincided with the classic experiments of Bernard Katz and colleagues on the quantal nature of neurotransmission at the frog neuromuscular junction and thus greatly strengthened the foundation for the quantal hypothesis of neurotransmitter release (8–10). Together, these findings led to the vesicle hypothesis, for example, that a single synaptic vesicle is the morphological correlate of a quantum of neurotransmitter (10).

Synaptic vesicle morphology and organization into functional pools

As judged by electron micrograph images, small synaptic vesicles have a clear core and are approximately 35–50 nm in diameter (11–13). By contrast, dense core vesicles, which are found in neuroendocrine cells and for the storage of neuropeptide transmitters in the nervous system, can be as large as 300–400 nm in diameter. Within the presynaptic terminal, synaptic vesicles seem to be morphologically identical at near nanometer resolution. However, three distinct functional pools can be identified based on the differential ability of synaptic vesicles to be recruited for fusion. The vesicles that are docked at the membrane surface in a region called the active zone and that have undergone a series of priming reactions to achieve fusion competence represent the readily releasable pool (RRP) (14). It is believed that release of neurotransmitter will occur predominantly (if not exclusively) from this vesicle pool. Because the synaptic vesicle priming reactions may be reversible, a small subset of the morphologically docked vesicles may exist in the unprimed state and therefore would be unavailable for release (15).

After the fusion of a synaptic vesicle, the RRP is refilled from the recycling pool of synaptic vesicles. For central nervous system synapses (e.g., synapses of hippocampal neurons), the recycling pool of synaptic vesicles consists of approximately 30 vesicles, approximately three to five times the number of RRP vesicles (15, 16). During repetitive synaptic stimulation, the rapid refilling of the RRP from the recycling pool sustains continuous neurotransmitter release. An overview of the synaptic vesicle cycle is shown in **Fig. 1**.

The third and largest synaptic vesicle pool is termed the reserve pool and does not contribute to neurotransmitter release under normal physiological conditions. It is proposed that reserve pool vesicles are only recruited with extremely intense extended bouts of synaptic stimulation, conditions under which the recycling pool of vesicles is depleted (17). When vesicle pool sizes are expressed as percentages of the total synaptic vesicle cluster, these percentages hold well across many synapse types and species. The RRP typically represents 1–2%, the recycling pool 10–20%, and the reserve pool 80–90% of the total vesicle cluster (18).

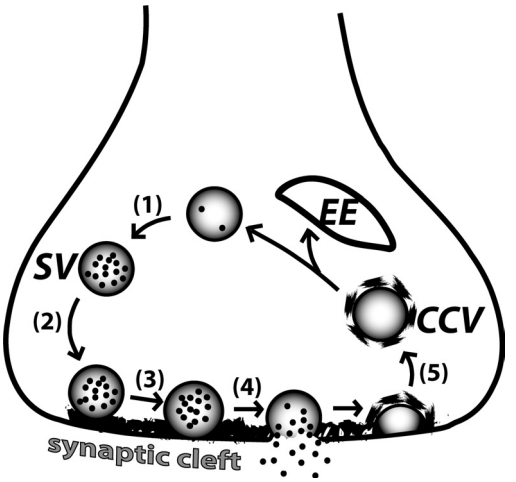

Figure 1 Overview of the synaptic vesicle cycle. (a) Within the presynaptic terminal, synaptic vesicles are filled with neurotransmitter by the action of specific vesicular neurotransmitter transporters. (b) Neurotransmitter-filled vesicles translocate to the active-zone membrane where they undergo docking. (c) Docked vesicles transition to a release-competent state through a series of priming or prefusion reactions. (d) Invasion of an action potential into the presynaptic terminal and subsequent calcium influx induces rapid fusion of the synaptic vesicle membrane with the terminal membrane, which thereby releases the neurotransmitter into the synaptic cleft. (e) Spent vesicles are internalized by clathrin-mediated endocytosis and are recycled for reuse, which thus completes the synaptic vesicle cycle. SV, synaptic vesicle; CCV, clathrin-coated vesicle; EE, early endosome. *NOTE*: The use of arrows indicates a temporal sequence of events. Physical translocation of synaptic vesicles is unlikely to occur between the docking and fusion steps.

The synaptic vesicle as an organelle for neurotransmitter storage and release

Glutamate, gamma amino butyric acid (GABA) and glycine, acetylcholine (Ach), and monoamines are examples of major small-molecule neurotransmitters in the nervous system. Although each neurotransmitter has a distinct structure and biological activity, all share the common feature of being concentrated into synaptic vesicles before release from the presynaptic bouton of the neuron. In this respect, the synaptic vesicle is an organelle specialized for storage and release of neurotransmitter. Furthermore, the synaptic vesicle contains numerous proteins (both transmembrane and vesicle-associated) that are key players in the biochemical reactions that lead to release of neurotransmitter. The very recent detailed molecular characterization of the synaptic vesicle as a model trafficking organelle, including some of the first available estimates of key synaptic vesicle protein copy numbers (13), reemphasizes the fact that the synaptic vesicle is center stage in the consideration of the neurotransmitter release process (**Table 1**).

Although the synaptic vesicle landscape is dominated by an array of proteins, it is of note that synaptic vesicles that contain different neurotransmitters are thought to have largely similar overall protein composition. For example, all synaptic vesicles

Table 1 Estimated copy number per vesicle and proposed functions for selected major synaptic vesicle proteins*

Synaptic vesicle protein	Copies per vesicle	Proposed function
Synaptobrevin/VAMP2	70	Priming
Synaptophysin	32	Vesicle recycling?
Synaptotagmin 1	15	Calcium sensor
Neurotransmitter transporter	9–14	Neurotransmitter loading
Rab3A	10	Docking?, priming
Synapsins	8	Regulation of vesicle mobility?
SV2	2	Priming, transporter?
Proton pump (V-ATPase)	1	Neurotransmitter loading

*Estimates of protein copy number per vesicle originally reported by Takamori et al. (13).

require proteins that are essential for membrane trafficking and fusion. The best-studied synaptic vesicle proteins include the transmembrane proteins synaptotagmin, synaptophysin, synaptobrevin (also referred to as vesicle associated membrane protein or VAMP), and synaptic vesicle protein 2 (SV2), as well as the peripherally associated synapsins and the Rabs that are attached through lipid modifications. The proton pump is also a key synaptic vesicle component and is critical for establishing the electrochemical gradient across the synaptic vesicle membrane (low pH in the lumen) to power neurotransmitter uptake into the synaptic vesicle lumen. The synaptic vesicle proton pump is unique in that only one copy per vesicle of this multiprotein complex exists, and this proton pump complex is by far the largest component of the synaptic vesicle (13, 18).

The neurotransmitter phenotype, (i.e., what type of neurotransmitter is stored and ultimately will be released from the synaptic bouton) is determined by the identity of the neurotransmitter transporter that resides on the synaptic vesicle membrane. Although some exceptions to the rule may exist; all synaptic vesicles of a given neuron normally will express only one transporter type and thus will have a defined neurotransmitter phenotype (this concept is enveloped in what is known as Dale's principle; see also Reference 19). To date, four major vesicular transporter systems have been characterized that support synaptic vesicle uptake of glutamate (VGLUT 1-3), GABA and glycine (VGAT), acetylcholine (VAChT), and monoamines such as dopamine, norepinephrine, and serotonin (VMAT 1 and 2). Vesicles that store and release neuropeptides do not have specific transporters to load and concentrate the peptides but, instead, are formed with the peptides already contained within.

Synaptic vesicle docking and priming reactions

Synaptic vesicle attachment or "docking" occurs in the active-zone region of the presynaptic terminal. This specificity for synaptic vesicle attachment implies a recognition mechanism between the synaptic vesicle and the active zone. In this context, some or perhaps all molecules responsible for synaptic vesicle docking might be expected to exhibit preferential enrichment or exclusive localization to the active-zone region. Despite intense efforts and numerous candidate molecules, the precise molecular mechanisms of synaptic vesicle docking remain poorly characterized at this time. The cytosolic protein Munc18 (which represents the major mammalian version of the sec1/munc18-like or SM family of proteins) is gaining acceptance as an important docking molecule (20), although no clear model for Munc18 function has emerged. Munc18 is proposed to provide the molecular link between the active-zone region and synaptic vesicles. It almost certainly links to the presynaptic membrane via a direct interaction with the N-terminal region of syntaxin (21); however, the interaction by which Munc18 can also link with synaptic vesicles has proven highly elusive. No additional putative interacting partners have been validated as essential for synaptic vesicle docking. This fact may indicate redundancy in synaptic vesicle docking mechanisms. Alternatively, very recent evidence that demonstrates that syntaxin is required for synaptic vesicle docking (22), despite prior evidence to the contrary (23, 24), may largely explain the notable lack of progress in unraveling synaptic vesicle docking mechanisms to date, although this controversial finding demands additional validation. Notably, neither syntaxin nor Munc18 are preferentially enriched in the active zone; therefore, it is likely that some other protein participates to impart the regional specificity. Such a role has been ascribed to the Munc13 protein, which is reported to be concentrated at active zones (25) and to function upstream of syntaxin in the docking of synaptic vesicles (22).

Priming can be defined as the transition of synaptic vesicles from the docked state into the state of release competence. The available evidence supports a critical role for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in the biochemical interactions that underlie synaptic vesicle priming. Our current understanding of intracellular membrane trafficking—including synaptic vesicle fusion—has developed from a general model known as the SNARE hypothesis (26). In this model, the formation of parallel-aligned α-helices between vesicle SNAREs and target-membrane SNAREs results in the formation of a remarkably stable SNARE complex (27). In the case of synaptic vesicle fusion, the synaptic vesicle protein synaptobrevin serves as the vesicle SNARE, whereas SNAP-25 (synaptosomal-associated protein of 25 kDa) and syntaxin on the presynaptic plasma membrane serve as the target-membrane SNAREs. These three

SNARE proteins assemble into a heterotrimeric SNARE complex (or core complex). The crystal structure of the core complex revealed a bundle of four α-helices, one each contributed by synaptobrevin and syntaxin and two contributed by SNAP-25 (28). The process of SNARE complex assembly proceeds from N-terminal to C-terminal direction in what is commonly referred to as a "zippering" action, which brings the C-terminal membrane anchors of the transSNAREs together (29). This action is proposed to force the closely apposed membranes together to initiate fusion.

The transition from unstructured monomeric SNAREs to the assembled SNARE complex likely proceeds by multiple sequential steps rather than at once, and zippering can only occur when all SNAREs are aligned in parallel. One model proposes that initially an acceptor complex forms between SNAP-25 and syntaxin on the plasma membrane (30–32). Only then would the acceptor complex interact with the vesicular SNARE synaptobrevin, aligning the N-terminal ends in parallel, first in a loose conformation, and then undergoing N-terminal to C-terminal zippering. The partially zippered SNARE complex is arrested in a partial fusion or prefusion state and awaits the influx of calcium to activate the neuronal calcium sensor and stimulate the completion of the fusion reaction. These processes are summarized in **Fig. 2**. One important open question is how SNARE zippering might become arrested before completion. Recent evidence supports a role for complexins at this step. Complexins are enriched in the presynaptic compartment, are crucial for highly synchronous evoked neurotransmitter release, and exhibit direct binding to the SNARE complex (33, 34). Although much evidence on complexin function is conflicting or controversial, most available evidence is consistent with a model in which complexins help to stabilize the partially zippered SNARE complex by direct binding but prevent full SNARE assembly and/or completion of fusion before the arrival of the calcium trigger (34–37). However, complexins do not merely function as inhibitory fusion clamps; it has been demonstrated that complexins are capable of both inhibitory and facilitating functions and thus are well suited to impart exquisite control in the final stages of calcium-dependent neurotransmitter release (34).

Although little doubt remains that the formation of the SNARE complex is the critical step in synaptic vesicle priming, several other proteins have been implicated as important regulators of the priming reactions. Two prominent examples are Munc18 and Munc13; these crucial proteins apparently function in both synaptic vesicle docking and priming. The available evidence is largely compatible with an essential role for both Munc18 and Munc13 in facilitating priming by regulating the proper assembly of the SNARE complex, which may be accomplished by the stabilization of the putative SNAP-25/syntaxin acceptor complex mentioned previously. It is unclear whether there is convergence exists between Munc18 and Munc13 in their respective modes of action in this context. To add another layer of complexity, Munc13 also has been shown to antagonize the action of a soluble protein called Tomosyn (38). Tomosyn contains a C-terminal SNARE motif through which it can form a tight complex with SNAP-25 and syntaxin and effectively deter the formation of the prototypical

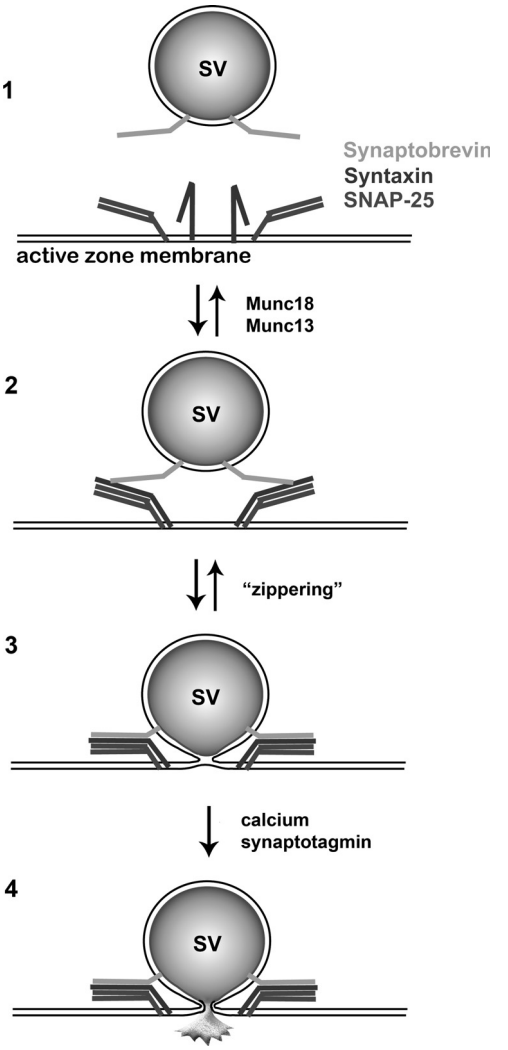

Figure 2 Biochemical reactions in the formation of the SNARE core complex. (a) Diagram of the vesicle SNARE synaptobrevin and the plasma membrane SNAREs syntaxin and SNAP-25. (b) Syntaxin must switch from a closed to an open conformation to assemble with SNAP-25 to form an acceptor complex for the vesicle SNARE synaptobrevin. Munc18 and Munc13 may function to stabilize (or facilitate the formation of) syntaxin/SNAP-25 heterodimers. (c) The initial heterotrimeric SNARE complex is in a loose conformation and transitions to a tight conformation via an N- to C-terminal ''zippering'' of the parallel-aligned SNARE motifs, which brings the synaptic vesicle and active-zone membrane together. Fusion is arrested at a prefusion stage in which it is proposed that only one leaflet of the fusing bilayers is merged. (d) The final fusion step is triggering rapidly by action potential invasion into the terminal, which leads to calcium influx and activation of the calcium sensor synaptotagmin. Calcium-bound synaptotagmin mediates the completion of fusion via calcium-dependent SNARE and phospholipid binding.

neuronal SNARE complex (39). Thus, Tomosyn serves as a negative regulator of vesicle fusion. Munc13 either prevents the formation of the Tomosyn-containing SNARE complexes or disrupts Tomosyn-containing SNARE complexes to liberate SNAP-25/syntaxin heterodimers, which then would become available to bind to synaptobrevin on the synaptic vesicle. Either model is again consistent with a positive regulatory role

for Munc 13 in synaptic vesicle priming. Other putative regulators of synaptic vesicle priming include RIM, Rab3, and SV2, although the exact mechanisms for how these proteins regulate priming are much less defined at this time.

Triggering of the synaptic vesicle fusion reaction

Once synaptic vesicle docking and priming is completed, the final triggering of vesicle fusion with the presynaptic terminal membrane occurs rapidly in response to action potential invasion of the terminal. This step is highly dependent on calcium ions, which enter the terminal through voltage-gated calcium channels (40–42). The idea that calcium entry into the terminal is a key step in the neurotransmitter release process formed the basis of what is known as the "calcium hypothesis" (43). Although the identity of the molecular calcium sensor for neurotransmission was not known at the introduction of the calcium hypothesis 40 years ago, it is now widely accepted that the calcium-dependent triggering of synaptic vesicle fusion is imparted by the synaptic vesicle protein synaptotagmin (44–46).

The work of Dodge and Rahamimoff (47) initially detailed the quantitative dependence of neurotransmitter release on external calcium concentration at the neuromuscular junction. The relationship was reported to be highly nonlinear with an approximately fourth-order cooperativity. Remarkably, these findings are in near perfect agreement with a more modern study of the relationship between neurotransmitter release and intracellular calcium concentration at a central nervous system synapse (48). Although the basis for the cooperativity of release is not yet clear, multiple calcium coordination sites exist with the tandem C2 domains of synaptotagmin, which suggests that this cooperativity of release may originate with the intrinsic calcium-binding ability of the synaptotagmin protein itself. This suggestion is supported by studies that show that mutations in the synaptotagmin C2 domains alter the apparent degree of cooperativity (45, 49).

How does synaptotagmin trigger synaptic vesicle fusion in response to calcium influx and binding? Synaptotagmin engages in calcium-stimulated binding of both SNARE complexes and membrane phospholipids (50–52). Although still debated, available evidence supports a model of synaptotagmin action in which calcium binding to the C2 domains causes membrane penetration and induction of positive membrane curvature. This buckling of the active-zone membrane locally reduces the energy barrier for fusion, which allows the fusion process to proceed to completion (53). A direct interaction of synaptotagmin to the SNARE complex is important in this model (calcium- dependent and/or -independent) to ensure that the local membrane buckling is targeted appropriately to the membrane beneath the vesicle; thus, the SNARE binding and phospholipid penetration activities of synaptotagmin likely work in concert to liberate neurotransmitter (52–55).

Vesicle fusion and neurotransmitter release in response to action potentials is termed evoked release (the main focus of this article); however, it is important to note that action potentials are not absolutely required for liberation of neurotransmitter. Spontaneous release events (also referred to as spontaneous miniature events or "minis" for short) occur in the absence of an overt stimulus (8). Evoked and spontaneous synaptic vesicle fusion likely share many commonalities, including the involvement of the same neuronal SNARE complex. However, perturbations to SNAREs can differentially affect evoked and spontaneous events, which suggests that the fusion reactions mediating evoked and spontaneous release events might differ to some extent (56–58).

Regulation of neurotransmitter release via modulation of the fusion pore

Although the molecular components of the neuronal fusion pore remain largely unknown, modulation of the fusion pore has been proposed as a mechanism for regulating release of neurotransmitter. Specifically, two modes of release can be summarized: 1) a classic full-fusion model in which the fusion pore completely dilates and the vesicle fully collapses into the plasma membrane of the active zone (59), which thereby releases a vesicle's full complement of neurotransmitter, and 2) a kiss-and-run model in which a narrow fusion pore is stabilized transiently but then closed rapidly (60). In the kiss-and-run model, the presence of a narrow fusion pore is hypothesized to restrict neurotransmitter efflux, and it thereby might reduce quantal size (61). The prevalence of kiss-and-run-type fusion events has been debated intensely; particularly, the prevalence of these events in central nervous system neurons (which we focus on here) has been debated, with some studies of hippocampal neurons estimating greater than 80% of all fusion events being of this type at low firing frequencies (62) and others reporting negligible kiss-and-run in the same preparation (63). The most recent and direct study to date reported only a 3% rate of occurrence for kiss-and-run at the specialized Calyx of Held synapse (64). Thus, although its frequency is widely debated, it is generally accepted that a nonclassic mode of fusion that is reasonably well described by the kiss-and-run model does in fact exist.

Does kiss-and-run-type fusion influence neurotransmitter release at central nervous system synapses? This topic is a current topic of research that is yet to be resolved; even the very existence of kiss-and-run exocytosis in the central nervous system is still controversial. It is worth noting that most current methods for detecting alterations to fusion pore size and/or conductance at central nervous system synapses have been unable to provide simultaneous direct measurements of neurotransmitter release. Thus, a marked absence of evidence exists to support the idea that fusion pore modulation can modulate the amount of neurotransmitter released during vesicle fusion. Of the studies that have achieved the technical feat of measuring neurotransmitter release during kiss-and-run-type fusion events, one study using a nonphysiological manipulation found no evidence for modulation of quantal size at glutamatergic central nervous system synapses (65), and another study detected, in a subset of release events, a predominant flickering fusion pore mechanism in which each flicker released only 25–30% of the total neurotransmitter contained within a vesicle at dopaminergic central nervous system synapses (66). It is likely that much of the controversy surrounding kiss-and-run can be resolved through

advancements in technical approaches for measuring neurotransmitter release.

Methods for Quantification of Neurotransmitter Release

To quantitate neurotransmitter release, we ideally would use approaches that can make noninvasive, selective, analytical measurements *in situ* with millisecond temporal resolution and micron or better spatial resolution. Each of these criteria has been fulfilled by one or more currently available methodologies. However, to date, no single technique is universally suited to all contexts of neurotransmitter-release quantification. Thus, compromises are made in the criteria that are less important to maximize the criteria that are most important to the biological question at hand. In choosing a technique, perhaps the first question is, what type of biological preparation is most appropriate for the study? Advantages of *in vitro* preparations make these preparations better suited for some lines of work, but advantages of *in vivo* preparations are better for others. Importantly, some techniques are better suited for some biological preparations than others. **Table 2** lists the preparations in which each of the techniques discussed below have been most commonly employed.

Radiolabeled neurotransmitters

One approach that has been used quite widely to quantitate neurotransmitter release employs radiolabeled (tritiated) neurotransmitter analogs (e.g., Reference 67). First, tissue is incubated in a buffer solution that contains tritiated neurotransmitter. During this time, the radiolabeled transmitter is taken up into cells by endogenous plasma-membrane transporters and packaged into vesicles by vesicular transporters. The tissue preparation then is rinsed in buffer to remove extracellular radiolabeled transmitter leaving only that which was taken up into cells. This stored transmitter is then released over time by exocytosis. To quantitate its release, the tissue is continuously perfused with buffer, and time-dependent aliquots are collected. Radioactivity is measured in the aliquots with a scintillation counter and is used as an index of endogenous neurotransmitter release. Rather than estimate absolute neurotransmitter release, this method is typically used to compare the relative release between two or more conditions.

Electrophysiological methods

Electrophysiological techniques have high temporal (millisecond) and spatial (micron) resolution. They are carried out at discrete electrodes and so obtain information from a single spatial location or from a finite number of locations if a multiple-electrode system is used. These recording techniques

Table 2 Tools for the quantification of neurotransmitter release and the biological preparations in which they have been used*

	In vitro		In vivo		Human
	Cell culture	Tissue slice	Anesthetized	Awake	
Interference reflection microscopy (IRM)	●	○	○	○	○
Total internal reflection fluorescence (TIRF)	●	○	○	○	○
Radiolabeled neurotransmitters	●	●	○	○	○
Fluorescent styryl dyes	●	●	○	○	○
SynaptopHlourins	●	●	○	○	○
False fluorescent neurotransmitters (FFNs)	●	●	○	○	○
Patch-clamp capacitance	●	●	○	○	○
Electrophysiological biosensors (sniffer patch)	●	●	○	○	○
Postsynaptic potentials	●	●	●	○	○
Amperometry	●	●	●	○	○
Fast-scan cyclic voltammetry	●	●	●	●	○
Electrochemical biosensors	●	●	●	●	○
High-speed chronoamperometry	○	○	●	●	○
Push-pull perfusion sampling	○	○	●	●	○
Microdialysis	○	○	●	●	●
Positron emission tomography (PET)	○	○	●	●	●
Magnetic resonance imaging (MRI)	○	○	●	●	●

*Note that this summary is not meant to impose limitations on the potential use of the techniques, but it highlights the scope in which they are routinely used currently.

use fairly standard electrophysiology recording resources and capabilities to measure neurotransmitter release, and so they are widely used.

Postsynaptic electrophysiological recordings detect the neurophysiological response of a target cell following release of neurotransmitter and represent the least deviation from a standard electrophysiological-recording experiment. These recordings are usually carried out at a patch-clamp electrode using a voltage clamp to measure postsynaptic currents (68), but the current clamp can be used if measurement of postsynaptic potentials is preferred. Selectivity of the responses for the neurotransmitter of interest can be achieved with appropriate pharmacological isolation by using a cocktail of antagonists for other neurotransmitters and/or with electrophysiological isolation (by manipulating the holding potential). Postsynaptic signals can be detected for both spontaneous and evoked neurotransmitter release events. The frequency of spontaneous postsynaptic events is often used as an index of neurotransmitter release. Information can also be obtained from the amplitude of postsynaptic responses; however, this metric encompasses both changes in neurotransmitter release (presynaptic effects) and changes in synaptic strength (postsynaptic effects). Presynaptic effects are usually inferred from experiments where postsynaptic responses are evoked by paired pulses of extracellular electrical stimulation separated by tens of milliseconds. The paired–pulse ratio (PPR), that is the amplitude of the response to the second pulse divided by that of the first, is believed to be a reflection of release probability, with low PPR signifying high release probability (argued to be because of depletion of ready-releasable vesicles on the first pulse; see Reference 5). Thus, changes in PPR are taken to indicate changes in the amount of neurotransmitter release that can be evoked by an electrical impulse.

During neurotransmitter release events from a cell, the surface area of its plasma membrane changes; it increases during fusion and decreases during subsequent endocytosis. Because the plasma membrane acts as an electrical capacitor, these dynamics can be detected as changes in the total membrane capacitance. Thus, membrane capacitance measurements with whole-cell or cell-attached patch clamp can be used to quantitate exocytosis (69). Using voltage clamp, a sine wave command signal is applied, and the current is measured with a lock-in amplifier to derive the instantaneous capacitance. With these recordings, individual exocytotic events can be detected. In cell-attached preparations, conductance of fusion pores that form within the patch can be measured simultaneously.

The electrophysiological biosensor or "sniffer patch" (70) uses an outside-out patch excised from a donor cell, which has high-density expression of ionotropic receptors for the neurotransmitter analyte of interest. Ideal donor cells are those in which the receptor has been stably overexpressed and low expression of other potential interfering receptors is detected. The electrode, incorporating the patch, is placed close to a putative release site, and channel conductance within the patch is measured in voltage-clamp mode. The neurotransmitter detection range of this technique is quite narrow, around the EC_{50} of the receptor, but it can be broadened somewhat by increasing the density of receptor expression in the patch. Nonetheless, the detection limit is, by definition, in the physiological range. The sensor response is nonlinear with analyte concentration, but it can give quantitative information with appropriate calibration.

Electrochemical methods

Electrochemical detection involves the induction of a change in redox state (electrolysis) by application of an electrical potential to an electrode (71). Compounds that can be readily detected by this means are termed electroactive. Under physiological conditions, these compounds tend to be in their reduced state in the nervous system because of the rich level of antioxidants (e.g., ascorbic acid) and, thus, can be oxidized by application of a positive potential to the electrode. The evolved electrons are detected at the electrode in the form of electrical current. This current is proportional to the number of electroactive molecules at the surface of the electrode, and therefore it is proportional to their concentration in the bulk solution. By implanting an electrode in the extracellular space close to the release site and detecting changes in the local (extracellular) concentration of the neurotransmitter, neurotransmitter release can be monitored. The key advantage of this approach is the high temporal resolution that can be in the millisecond domain. Neurotransmitters that can be detected this way include dopamine, norepinephrine, epinephrine, serotonin, and melatonin.

Several variants of this technology differ by the voltage command waveform applied to the electrode to induce electrolysis. The simplest waveform is a constant direct-current potential. This form is known as constant-potential amperometry. This technique yields a constant readout of neurotransmitter fluctuations in real time and provides the fastest available chemical monitoring. For example, using constant-potential amperometry, data has been acquired in the high microsecond range that is sufficient to resolve release during multiple partial fusion events (flickering) (66). However, the disadvantage of this type of electrochemical detection is that it lacks chemical selectivity. Thus, its use is limited to environments where the identity of the analyte is predictable (see Reference 71).

Another commonly used waveform is a square wave where the electrode is held at a nonoxidizing potential and transiently pulsed to an oxidizing potential. An example of this form is high-speed chronoamperometry, which uses oxidizing pulses that typically last for around 100 ms and are repeated each second. This method provides information (current) both on the oxidation of compounds at the surface of the electrode and on the reduction of the oxidized material. This additional information that is obtained during each measurement aids in the identification of the analyte.

To improve chemical selectivity, a triangular input waveform can be used that separates compounds into resolvable peaks. This form—cyclic voltammetry—can be carried out with high temporal resolution using high scan rates to allow the waveform to be completed in a short time. In fast-scan cyclic voltammetry (also known as fast cyclic voltammetry), waveforms last around 10 ms, and measurements are typically made every 10–200 ms.

The newest generation of electrochemical-based biological detection devices is the biosensors. These devices combine the high spatial and temporal resolution of an electrochemical microsensor with a biologically selective recognition element. In

current devices, this element is most often an enzyme that is selective for the neurotransmitter of interest. The enzyme exists in a polymer layer that is embedded on an electrochemical probe. When the analyte binds to the enzyme, a chemical coreaction is initiated that generates an electroactive reporter molecule. The reporter molecule is detected by the electrochemical probe using constant-potential amperometry and thus transduces the biological signal to an electrochemical signal. This approach has three main advantages over other electrochemical sensors. First, the range of possible analytes is not limited to those that are electroactive. In fact, it is advantageous for the analyte not to be electroactive so that its direct detection at the electrochemical sensor does not interfere with detection of the reporter molecule. Second, the selectivity of the probe is conferred by a recognition element that can be highly specific. Third, this technology lends itself to upgradeability; new recognition elements can be incorporated into probes as they are developed.

Optical methods

Optical methods offer the benefit of extremely high spatial resolution, in the nanometer range. Moreover, unlike electrode-based techniques (e.g., electrophysiological and electrochemical methods) these measurements can be made over a wide area; that is, rather than obtaining a single time-dependent measurement, multiple parallel processes can be monitored simultaneously at discrete spatial loci (e.g., Reference 72). Currently, optical methods for quantification of neurotransmitter release are not widely used *in vivo* because of insufficient deep-brain optical access. However, a rapidly growing movement is working to develop these tools for use in systems neuroscience (e.g., using techniques that can penetrate deep enough to measure from superficial cortical layers or fiber optics to access deeper regions).

Fluorescent styryl dyes such as FM1-43 have been used to approximate neurotransmitter release by measuring rates of exocytosis (16, 72, 73). These dyes reversibly label endosomal membranes and can be taken up into intracellular synaptic vesicles during endocytosis in systems in which vesicle recycling takes place. Typically, tissue is incubated in the fluorescent dye and then stimulated to promote vesicle cycling and therefore uptake of the dye. The preparation then is washed in fresh buffer to remove dye that remained extracellular. Using fluorescent microscopy, vesicle dynamics can be tracked. Neurotransmitter release is estimated from the rate of destaining (because of exocytosis) usually during stimulation.

Optical imaging of neurotransmitter release has also been conducted using genetically expressed fluorescent proteins that are fused to vesicular proteins. The prototypical fluorescent fusion protein for this method is synaptopHlourin (74). SynaptopHlourin is a pH-sensitive green fluorescent protein mutant (ecliptic pHlourin) fused to the (lumenally exposed) C-terminus of synaptobrevin; it can be introduced to a cell by transfection or constitutively expressed in transgenic animals. At low pH, the fluorescence of the pHlourin is quenced by more than ten fold. Thus, before neurotransmitter release from a synaptic vesicle that expresses a synaptopHlourin, the pHlourin moiety resides in the vesicular lumen where it has minimal fluorescence because of the acidic environment. However, following fusion, extracellular fluid enters through the pore, diluting the proton concentration (i.e., increasing pH) and permitting the pHlourin to fluoresce. The fluorescence is quenced again after endocytosis as the recycled vesicle is acidified. Thus, this system produces transient fluorescence that indicates the time after fusion, before endocytosis.

Very recently, neuroscientists and chemists from Columbia University have collaborated to develop false fluorescent neurotransmitters (FFNs) (75). These molecules are hybrids between neurotransmitter analogs and fluorophores. The goal of this initiative is to produce fluorescent compounds that are substrates for neurotransmitter plasma and vesicular transporters so that their compartmentalization parallels that of endogenous neurotransmitters. In the context of quantification of neurotransmitter release, this method has the advantage over styryl dyes and synaptopHlorins in that it tracks neurotransmitter dynamics *per se* rather than monitoring exocytosis. Thus, it has the potential to measure transmitter kinetics during partial fusion, for example, in kiss-and-run, and can distinguish between synaptic vesicles that contain neurotransmitter and synaptic vesicles that are "blanks." Furthermore, unlike imaging styryl dyes, this method can be used to measure neurotransmitter release in systems in which vesicle recycling is not used, such as neurosecretory cells or cells that release neuropeptides.

Forms of reflection microscopy, at the interface between the plasma membrane of a cell and a glass coverslip, can also be used to track exocytosis and the events leading up to it. In total internal reflection fluorescence (TIRF) microscopy (76), a laser is directed toward the interface at an incident angle greater than the critical angle for total reflection of the light. An evanescent wave is generated that penetrates a small distance into the cell (about half the excitation wavelength: $\sim$200 nm) that can excite fluorophores. Because the excitation light is reflected at a high incident angle, interference with detection of emitted light is minimal, and the signal-to-noise ratio of this technique is high. If vesicles are fluorescently labeled, then their mobility can be tracked while they are close to the plasma membrane during docking, priming, and exocytosis (76). Interference reflectance microscopy (IRM) also has the capacity to monitor vesicles close to the plasma membrane (77). However, this technique has the advantage that a fluorophore is not required.

Sampling methods

Although most methods for quantifying neurotransmitter release discussed here make *in situ* measurements, some advantages exist when moving material to a remote location for analysis, for example, sampling. The primary advantage is that this methodology is not constrained to a single analytical tool, and detection schemes can be tailored to the needs of the particular experiment and to measure multiple analytes simultaneously. In fact, any tools of analytical chemistry can be used, including those that cannot easily be miniaturized into an *in situ* device, for example, gas chromatography or mass spectroscopy. Sampled material is often analyzed offline, but online analysis can be achieved by coupling a detector to the sampling-device outlet if desired.

Microdialysis sampling (78) is conducted using a probe that has an inlet and outlet tube joined via a semipermeable membrane (often a concentric design with the inlet inside the outlet tube). The membrane has a molecular-weight cutoff high enough that it is permeable to small-molecule and peptide neurotransmitters. Buffer (usually artificial cerebrospinal fluid) is perfused through the inlet tube past the membrane. When the probe is implanted in the brain, neurotransmitters in the extracellular space diffuse across the membrane into the probe down their concentration gradient. An equilibrium between the extracellular and intraprobe neurotransmitter concentration is accomplished, which is dependent on the flow rate, membrane size, and other factors. The ratio between the analyte concentration inside the probe and that on the outside at equilibrium is the relative recovery. Under most experimental conditions, the relative recovery is below 100%, and thus the neurotransmitter concentration in the dialysate is an underestimate of the absolute extracellular concentration. To better measure the absolute basal transmitter level, the no net flux method can be used in which the analyte of interest is perfused through the probe at different concentrations. When the analyte concentration is higher in the inlet than outside the probe, a net flux moves out of the probe, and the concentration will be lower in the outlet than the inlet. Conversely, when the analyte concentration is lower in the inlet than outside the probe, a net flux moves into the probe (and the concentration will be higher in the outlet than the inlet). Therefore, the point of no net flux (which can be interpolated if not measured directly) represents the condition in which the inlet concentration matches the concentration of the analyte outside the probe.

Another means of sampling is push–pull perfusion (79) using a probe with discrete inlet and outlet tubes. With this method, a small amount of cerebrospinal fluid is pulled directly from the brain through the outlet tube and replaced with artificial cerebrospinal fluid administered via the inlet tube. This approach has greater spatial resolution than microdialysis; and because cerebrospinal fluid is collected directly, no concern develops about incomplete recovery.

One of the largest drawbacks of these techniques is the time it takes to collect sufficient material for an analytic sample. Typically, this time has been on the order of tens of minutes, and thus these techniques are best suited for measuring steady-state levels of neurotransmitter and slow signal changes. However, in recent years, the temporal resolution has been improved significantly to the level of seconds by using capillary and microfluidic devices for sample collection combined with online separation and detection (79, 80).

Neuroimaging

Neuroimaging is the least invasive means to measure neurotransmitter release and can be used in living animals or humans without surgical procedures. These techniques permit monitoring over a large area (e.g., the whole brain) in three dimensions with millimeter spatial resolution and temporal resolution in seconds to minutes (81).

Positron emission tomography (PET) detects and spatial localizes radioactive sources, and it can be used to quantitate neurotransmitter release by measuring displacement of radiolabeled ligands (usually antagonists) from neurotransmitter receptors. The radioligand is administered systemically and is sequestered in tissue by selective binding to its receptor. On radioactive decay, a positron is emitted that collides with an electron (annihilation) and produces two photons (gamma rays) that travel in opposite directions. The scanner has a ring of gamma detectors that senses the arrival time of the photons. From the position in the ring and the difference in time of flight between the photons, the location of the annihilation within the plane of the ring can be calculated. The third dimension can be reconstructed by conducting serial measurements in adjacent "slices." Thus, PET provides a spatial map of the radioligand throughout the brain, which can be updated every few minutes. When an increase in endogenous neurotransmitter release occurs, the radioligand is displaced from receptors, and the tissue radioactivity level is reduced. Note that because this method relies on competitive displacement of a receptor-bound radioligand, increases but not decreases in endogenous neurotransmitter levels (i.e., increases but not decreases in the rate of ongoing neurotransmitter release) can be detected.

Magnetic resonance imaging (MRI) is included here because it is the least invasive method for gleaning information about neurotransmission in living humans. However, it should be noted that the inferences about neurotransmitter release are extremely indirect. This technique is used to measure, on a timescale of seconds, local hemodynamic changes that are assumed to correlate with neurotransmission. The experiment is carried out in a high-level magnetic field (>1 tesla). Excitation by radio-frequency pulses alters the spin axis of hydrogen nuclei (protons; including those in water), which can be measured as changes in the field strength. The dynamics of relaxation of the proton spin axis back to equilibrium follows two time constants that account for spin–lattice relaxation (T1) and spin–spin relaxation (T2) and depend on tissue molecular interactions. Spin–lattice relaxation is the realignment of the proton spin axis in the longitudinal axis of the magnetic field (z-axis), and spin–spin relaxation is that in the transverse (xy) plane. With the appropriate pulse sequence, a blood–oxygen-level dependent (BOLD) signal, the hemodynamic response, can be extracted from the T2 signal and is used to infer regional brain activity. These types of measurements are typically made during sensory stimulation, in behavioral or cognitive tasks (functional MRI; fMRI) or after administration of pharmacological agents (pharmacologic MRI; phMRI).

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See Also

Membrane Trafficking
Membrane Fusion, Mechanisms of
Neurotransmitter: Production and Storage
Neurotransmitter: Uptake and Degradation
Neurotransmission, Chemical Events in
Synaptic Chemistry