CHAPTER

12

Synaptic Transmission and Cellular Signaling: An Overview

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OUTLINE

SYNAPTIC TRANSMISSION

Chemical transmission between nerve cells involves multiple steps

Until the late nineteenth century, many physiologists believed that there were direct physical connections between nerves and that an impulse from one nerve was communicated to another through a direct physical connection. However, studies by Golgi, Ramon y Cajal and others convinced many histologists that most connections, which we now know as synapses, were close but not continuous. The pioneering work of Oliver and Shäfer, of Langley and of Elliot, beginning in the 1890s, provided data that raised the possibility of chemical transmission between nerves. Chemical transmission was convincingly demonstrated in the historic experiments of Otto Loewi. He electrically stimulated the vagus nerve of an isolated frog heart to decrease the strength and rate of contractions. The bathing solution caused a decrease in the strength and rate of contractions when subsequently applied to a second heart. We now know that the inhibition was caused by the neurotransmitter acetylcholine (ACh), which had been released by the nerve

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terminals of the vagus nerve. (See Davenport, 1991 for an entertaining and excellent review of the early history of chemical transmission.)

Chemical transmission is the major means by which nerves communicate with one another in the nervous system. The pre- and postsynaptic events are highly regulated and subject to use-dependent changes that are the basis for plasticity and learning in the CNS. Although direct electrical connections also occur, these account for transmission of information between nerves only in specialized cases.

Chemical transmission requires the following steps:

1. synthesis of the neurotransmitter in the presynaptic nerve terminal
2. storage of the neurotransmitter in secretory vesicles
3. regulated release of neurotransmitter in the synaptic space between the pre- and postsynaptic neurons
4. the presence of specific receptors for the neurotransmitter on the postsynaptic membrane, such that application of the neurotransmitter to the synapse mimics the effects of nerve stimulation
5. a means for termination of the action of the released neurotransmitter

An overview of some of the processes involved in synaptic transmission is shown in Figure 12-1. Many of the processes are discussed below or in other chapters of this book. Many different types of substances are neurotransmitters. "Classical" neurotransmitters, such as ACh (Chap. 13) and norepinephrine (NE) (Chap. 14), are low-molecular-weight substances that have no other function but to serve as neurotransmitters. The predominant excitatory neurotransmitter in the brain, glutamate, and the inhibitory neurotransmitters, GABA in brain and glycine in the spinal cord, are common amino acids that participate in multiple metabolic cycles (Box 12-1 and Chap. 17). They can function as neurotransmitters because the membranes of secretory vesicles in glutamatergic and glycinergic nerve terminals have specific transport

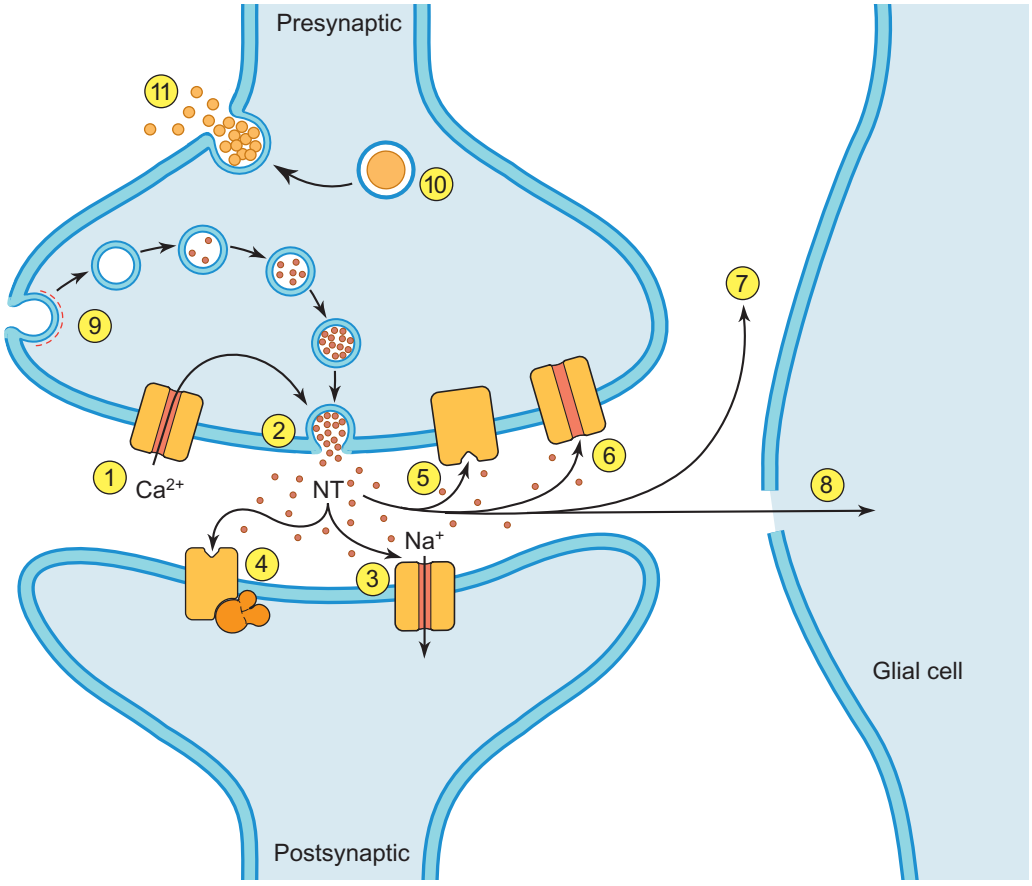


FIGURE 12-1 **Depolarization opens voltage-sensitive Ca^{2+} channels in the presynaptic nerve terminal (*1*).** The influx of Ca^{2+} and the resulting high Ca^{2+} concentrations at active zones on the plasmalemma trigger (*2*) the exocytosis of small synaptic vesicles that store neurotransmitter (NT) involved in fast neurotransmission. Released neurotransmitter interacts with receptors in the postsynaptic membrane that either couple directly with ion channels (*3*) or act through second messengers, such as (*4*) G-protein–coupled receptors. Neurotransmitter receptors, also in the presynaptic nerve terminal membrane (*5*), either inhibit or enhance exocytosis upon subsequent depolarization. Released neurotransmitter is inactivated by reuptake into the nerve terminal by (*6*) a transport protein coupled to the Na^+ gradient, for example, dopamine, norepinephrine, glutamate and GABA; by (*7*) degradation (acetylcholine, peptides); or by (*8*) uptake and metabolism by glial cells (glutamate). The synaptic vesicle membrane is recycled by (*9*) clathrin-mediated endocytosis. Neuropeptides and proteins are stored in (*10*) larger, dense core granules within the nerve terminal. These dense core granules are released from (*11*) sites distinct from active zones after repetitive stimulation.

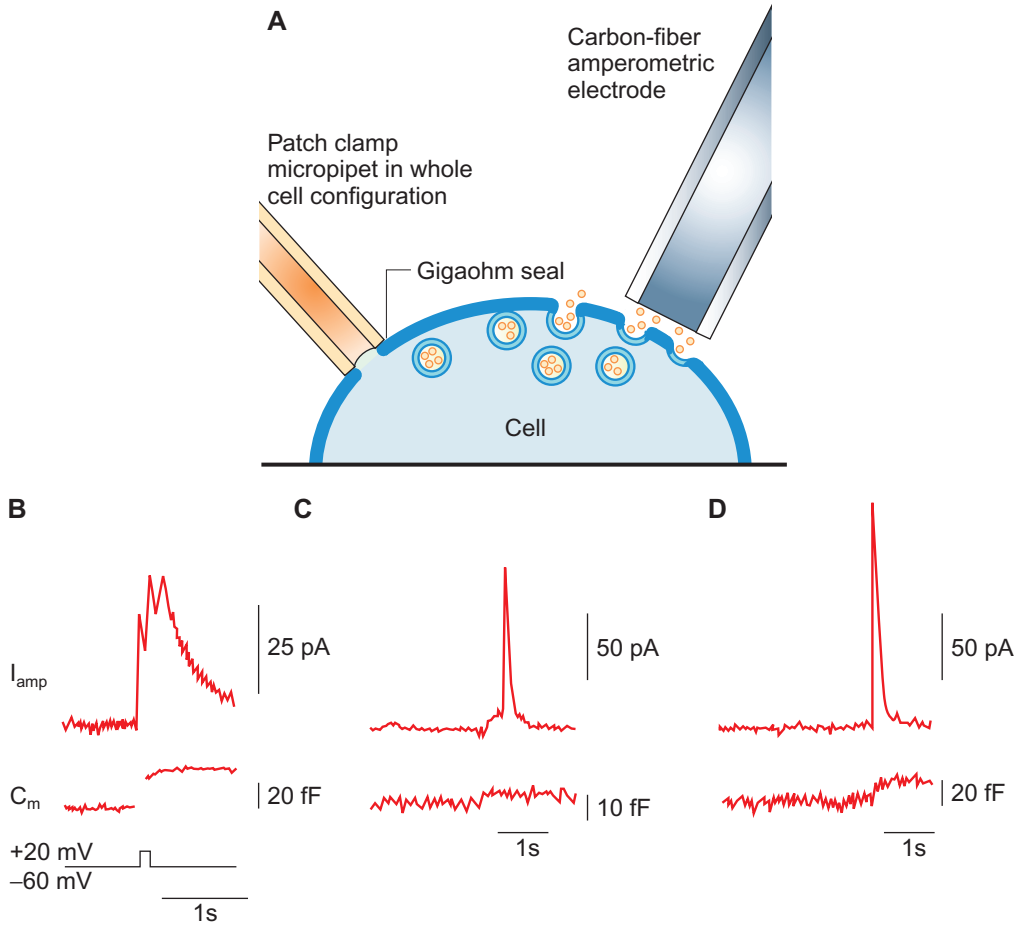


FIGURE 12-2 **Secretory events monitored by simultaneous amperometric (I_{amp}) and capacitance (C_m) measurements demonstrate typical patterns of release**. (**A**) Configuration of the recording setup. (**B**) Wide amperometric response composed of multiple spikes due to the fusion of several secretory vesicles triggered by a 50 ms depolarization to +20 mV from a holding potential of −60 mV. From the magnitude of the capacitance response (24 fF), it is estimated that between 2 and 24 vesicles fused with the plasmalemma. The exact number of vesicles that fused is not known since there is a distribution in their size. The amperometric response shows at least three to five discernible peaks that lag the capacitance step. However, the broad amperometric response (**B**) is likely to be composed of many more spikes. Two particularly large, isolated fusion events are shown in **C** and **D**. A clear lag between the fusion of a vesicle and the main spike of release can sometimes be observed, resulting in a foot before the spike (**C**); however, it is not always present (**D**). (Adapted with permission, Robinson et al., 1995.)

systems that concentrate and store these amino acids so that they can be released by exocytosis in a highly regulated manner. Aminergic neurotransmitters and ACh also enter synaptic vesicles through specific transport proteins. Synaptic vesicles have an acidic interior, pH ~5.5, which is maintained by a vacuolar-type proton-translocating ATPase (see Chap. 3). The uptake of low-molecular-weight neurotransmitters is coupled via the transporters to the electrochemical H^+ gradient (for review, see Njus et al., (1995)).

Neurotransmitter release is a highly specialized form of the secretory process that occurs in virtually all eukaryotic cells

The fundamental similarity between the events in the nerve terminal that control neurotransmitter release and the ubiquitous vesicular trafficking reactions in all eukaryotic cells is described in Chapter 7. This similarity has important implications for the biochemistry of synaptic transmission. Many of the proteins essential for constitutive secretion and endocytosis in yeast and mammalian cells are similar to those involved in the presynaptic events of synaptic transmission (Chap. 7).

Peptides and proteins can also be released from nerve terminals. Their biosynthetic and storage processes are similar to those in other protein secretory cells (Palade, 1975). They utilize the endoplasmic reticulum, Golgi and trans-Golgi network, which are present in the cell body but not in the nerve terminal. The peptide- and protein-containing vesicles must be transported into the nerve terminal by axonal transport (Chap. 8). Examples of peptide neurotransmitters include substance P, thyrotropin-releasing hormone (TRH), vasopressin, oxytocin, enkephalins and endorphins

(endogenous opiate-like agonists), brain-derived neurotrophic factor (BDNF), vasoactive intestinal peptide (VIP) and luteinizing hormone–releasing hormone (LHRH) (Chap. 20). The interior of mature secretory granules in neuroendocrine cells in the regulated pathway has a pH of 5.3 to 5.5, similar to the pH in synaptic vesicles. The low pH influences intravesicular protein processing and the conformation of the stored proteins, as well as the transport of other substances into the granules.

A variety of methods have been developed to study exocytosis

Neurotransmitter and hormone release can be measured by electrical effects of released neurotransmitter or hormone on postsynaptic membrane receptors, such as the neuromuscular junction (NMJ) (see below), and directly by biochemical assay. Another direct measure of exocytosis is the increase in membrane area due to the incorporation of the secretory granule or vesicle membrane into the plasma membrane. This can be measured by increases in membrane capacitance (C_m). C_m is directly proportional to membrane area and is defined as: $C_m = QA_m/V$, where C_m is the membrane capacitance in farads (F), Q is the charge across the membrane (coulombs), V is voltage (volts) and A_m is the area of the plasma membrane (cm^2). The specific capacitance, Q/V, is the amount of charge that must be deposited across $1\,cm^2$ of membrane to change the potential by 1 volt. The specific capacitance is mainly determined by the thickness and dielectric constant of the phospholipid bilayer membrane and is similar for intracellular organelles and the plasma membrane. It is approximately $1\,\mu F/cm^2$. Therefore, the increase in plasma membrane area due to exocytosis is proportional to the increase in C_m.

The electrophysiological technique used to measure changes in membrane capacitance is the patch clamp (Neher & Marty, 1982) in the whole-cell recording mode, where the plasma membrane patch in the pipette is ruptured. In another configuration of the patch clamp, the plasma membrane patch is maintained intact. In this case, small currents due to the opening of individual channels can be measured in the membrane patch. The whole-cell patch clamp technique establishes a high resistance seal between the glass rim of the micropipette and the plasma membrane that allows low-noise, high-sensitivity electrical measurements across the entire plasma membrane. An example of the use of membrane capacitance to measure exocytosis in chromaffin cells is shown in Figure 12-2 (Robinson et al., 1995).

Sensitive electrochemical techniques have also been developed to directly measure the release of oxidizable neurotransmitters such as catecholamines (CAs) and serotonin (5-hydroxytryptamine; 5-HT). Current flows in the circuit when the potential of the electrode is positive enough to withdraw electrons from, that is, oxidize, the released neurotransmitter. The technique is very sensitive and readily detects the release of individual quanta of neurotransmitter resulting from the fusion of single secretory granules (Fig. 12-2).

A variety of different types of tissue preparations are used to study neurosecretion and synaptic transmission. A classical preparation is the frog NMJ (discussed below). The brain slice has been used for many years for biochemical studies of CNS metabolism and is a useful preparation for electrophysiological studies of synaptic transmission in the CNS. Slices can be oriented to maintain the local neuronal circuitry and can be thin, ~0.3 mm, to minimize anoxia. The transverse hippocampal slice is widely used as an electrophysiological preparation to study synaptic plasticity (see Chap. 56). Primary cultures of neurons from selected CNS areas and sympathetic ganglia are also frequently used. They permit excellent visual identification of individual neurons and control of the extracellular milieu, but the normal neuronal connections are disrupted.

Gentle homogenization of brain tissue results in suspensions of intracellular organelles and pinched-off nerve terminals, *synaptosomes*. Homogenization shears off nerve terminals from axons, especially in brain regions with clearly defined anatomical layers, such as the cerebral cortex and hippocampus. Synaptosomes can be partially separated from other organelles by centrifugation techniques. Each of these remarkable structures is 0.5 to $1.0\,\mu m$ in diameter, contains hundreds of synaptic vesicles and one or more mitochondria, and is often associated with postsynaptic membrane fragments. Synaptosomes remain functional for several hours and can be used to study biochemical events, including energy and Ca^{2+} metabolism, neurotransmitter synthesis, transport and secretion. A related preparation is the neurosecretosome, from the posterior pituitary. These nerve terminals originate in the hypothalamus and contain vasopressin and oxytocin in large dense core granules. They are obtained in high purity from the neurohypophysis, which does not contain cell bodies. Neurosecretosomes are somewhat larger than synaptosomes and can be used for biochemical and patch clamp studies.

Several types of cells related to sympathetic neurons can be maintained and studied in tissue culture. Adrenal medullary chromaffin cells have the same precursor cells as postganglionic sympathetic neurons. These excitable neuroendocrine cells store, in large dense core granules called chromaffin granules, epinephrine (EPI) or NE, together with ATP, and a variety of proteins (chromogranins, opiate peptides and precursors), including enzymes for NE and epinephrine synthesis (dopamine β-hydroxylase) and protein processing (prohormone convertases). Relatively pure primary cultures can be prepared by collagenase digestion of bovine adrenal glands followed by cell-purification techniques. Various aspects of neurotransmitter metabolism and secretion have been extensively studied with these cells. They are amenable to both biochemical and electrophysiological experiments. A clonal cell line, PC12, is derived from a rat pheochromocytoma, a tumor of the adrenal medulla. Upon incubation with nerve growth factor (NGF), PC12 cells differentiate within days into neurons with axons and terminals (Chap. 29). Thus, they are used not only for biochemical and secretion studies but also for investigation of neuronal differentiation.

The neuromuscular junction is a well-defined structure that mediates the release and postsynaptic effects of acetylcholine

The first detailed studies of synaptic transmission were performed at the NMJ. The NMJ is a beautiful example of

how structure and function are intimately entwined. The myelinated axon originating from the motor neuron in the spinal cord forms unmyelinated terminals that run longitudinally along the muscle fiber. Specialized transverse release sites, or active zones, occur periodically along the terminals and are oriented opposite invaginations of the postsynaptic membrane (Fig. 12-3). There are approximately 300 active zones per NMJ. The active zones in the nerve terminal display a cloud of clear vesicles, 50 to 60 nm in diameter, that contain ACh. The active zone contains aggregates of proteins (active zone material, AZM) composed of discrete structures that link the docked granules to the plasma membrane and probably play a role in exocytosis [6a]. There are approximately 500,000 vesicles in all of the active zones at one NMJ. It is estimated that on the average a vesicle contains 20,000 ACh molecules. A small subset of the vesicles is attached in rows to the presynaptic membrane (Fig. 12-3A, B). These are thought to be docked vesicles that are able to undergo exocytosis upon Ca^{2+} influx. In freeze fracture, these rows coincide with rows of intramembrane particles that may be Ca^{2+} channels (Fig. 12-3C). Ca^{2+} entry that occurs upon stimulation of the nerve causes exocytosis that is seen as pits in freeze-fracture micrographs (Fig. 12-3D) or as "omega" figures in thin-section electron microscopy (Fig. 12-4). The vesicle membranes in the nerve terminal are recycled by endocytosis (see below).

The postsynaptic membrane opposite release sites is also highly specialized, consisting of folds of plasma membrane containing a high density of nicotinic ACh receptors (nAChRs). Basal lamina matrix proteins are important for the formation and maintenance of the NMJ and are concentrated in the cleft. Acetylcholinesterase (AChE), an enzyme that hydrolyzes ACh to acetate and choline to inactivate the neurotransmitter, is associated with the basal lamina (see Chap. 13).

Quantal analysis defines the mechanism of release as exocytosis

Stimulation of the motor neuron causes a large depolarization of the motor end plate. In 1952, Fatt & Katz, observed that spontaneous potentials of approximately 1 mV occur at the motor end plate. Each individual potential change has a time course similar to the much larger evoked response of the muscle membrane that results from electrical stimulation of the motor nerve. These small spontaneous potentials were therefore called miniature endplate potentials (MEPPs). Because the MEPPs are reduced by the nicotinic antagonist d-tubocurarine and increased in amplitude and duration by the AChE inhibitor prostigmine, it was concluded that they are initiated by the release of ACh. Because the potential changes are too large to be accounted for by the interaction of individual molecules of ACh with the endplate, Fatt and Katz postulated that they reflect the release of packets, or *quanta*, of ACh molecules from the nerve terminal.

A "curious effect" was observed by Fatt and Katz (1952): when the Ca^{2+} concentration is reduced and the Mg^{2+} increased, the evoked endplate potential (EPP) is diminished without altering the size of the spontaneous MEPPs. With sufficiently low Ca^{2+}, the evoked EPP is similar in size to MEPPs and varies in a stepwise manner. A single nerve impulse results in either no EPP or EPPs the approximate size of one, two, three or more MEPPs in an apparently random manner. The results of this type of experiment are shown in Figure 12-5. The frequency histogram shows that the amplitudes of evoked potentials are clustered in multiples of the mean spontaneous MEPP value. Statistical analysis (Del Castillo & Katz, 1954) demonstrates that the release is a random process described by a Poisson distribution. Each event is unaffected by the preceding events. The model assumes n release sites capable of responding to a nerve impulse, each with a probability, p, of releasing a quantum of ACh. The mean number of quanta (m), or quantal content, released per nerve impulse is $m = np$. For a Poisson distribution, p must be small, <0.05, and n large, >100. The probability of evoked release of x quanta is $P_x = (m^x/x!)e^{-m}$. (See Martin, 1977 for a review of the Poisson distribution in the analysis of synaptic transmission.)

One critical test for the validity of the Poisson distribution as a description of release in the presence of reduced Ca^{2+} was the excellent agreement of two measures of m. One was derived empirically; The other was derived from the Poisson equation and the observed probability of no response, or failures, upon nerve stimulation.

A more stringent test of the model is its ability to predict the histogram in Figure 12-5.

The quantal size m differs for different types of synapses. For a single impulse at the NMJ, 100 to 300 quanta are released. The large number of quanta that are released during a single impulse reflects the need for a large safety factor in the all-or-none response of muscle contraction. Where integration of inputs is important, quantal size is often less. At single terminals in sympathetic ganglia, at inhibitory and excitatory inputs on spinal motor neurons and at individual boutons of cultured hippocampal neurons, m is 1 to 3.

Ca^{2+} is necessary for transmission at the neuromuscular junction and other synapses and plays a special role in exocytosis

In most cases in the CNS and PNS, chemical transmission does not occur unless Ca^{2+} is present in the extracellular fluid. Katz & Miledi (1967) elegantly demonstrated the critical role of Ca^{2+} in neurotransmitter release. The frog NMJ was perfused with salt solution containing Mg^{2+} but deficient in Ca^{2+}. A twin-barrel micropipette, with each barrel filled with either 1.0 M $CaCl_2$ or NaCl, was placed immediately adjacent to the terminal. The sodium barrel was used to depolarize the nerve terminal electrically and the calcium barrel to apply Ca^{2+} ionotophoretically. Depolarization without Ca^{2+} failed to elicit an EPP (Fig. 12-6A). If Ca^{2+} was applied just before the depolarization, EPPs were evoked (Fig. 12-6B). In contrast, EPPs could not be elicited if the Ca^{2+} pulse immediately followed the depolarization (Fig. 12-6C). EPPs occurred when a Ca^{2+} pulse as short as 1 ms preceded the start of the depolarizing pulse by as little as 50 to 100 μs. The experiments demonstrated that Ca^{2+} must be present when a nerve terminal is depolarized in order for neurotransmitter to be released.

The normal extracellular Ca^{2+} concentration is approximately 2 mM. The basal cytosolic Ca^{2+} concentration is 0.1 μM or less. In nerve terminals, the rise of intracellular Ca^{2+}

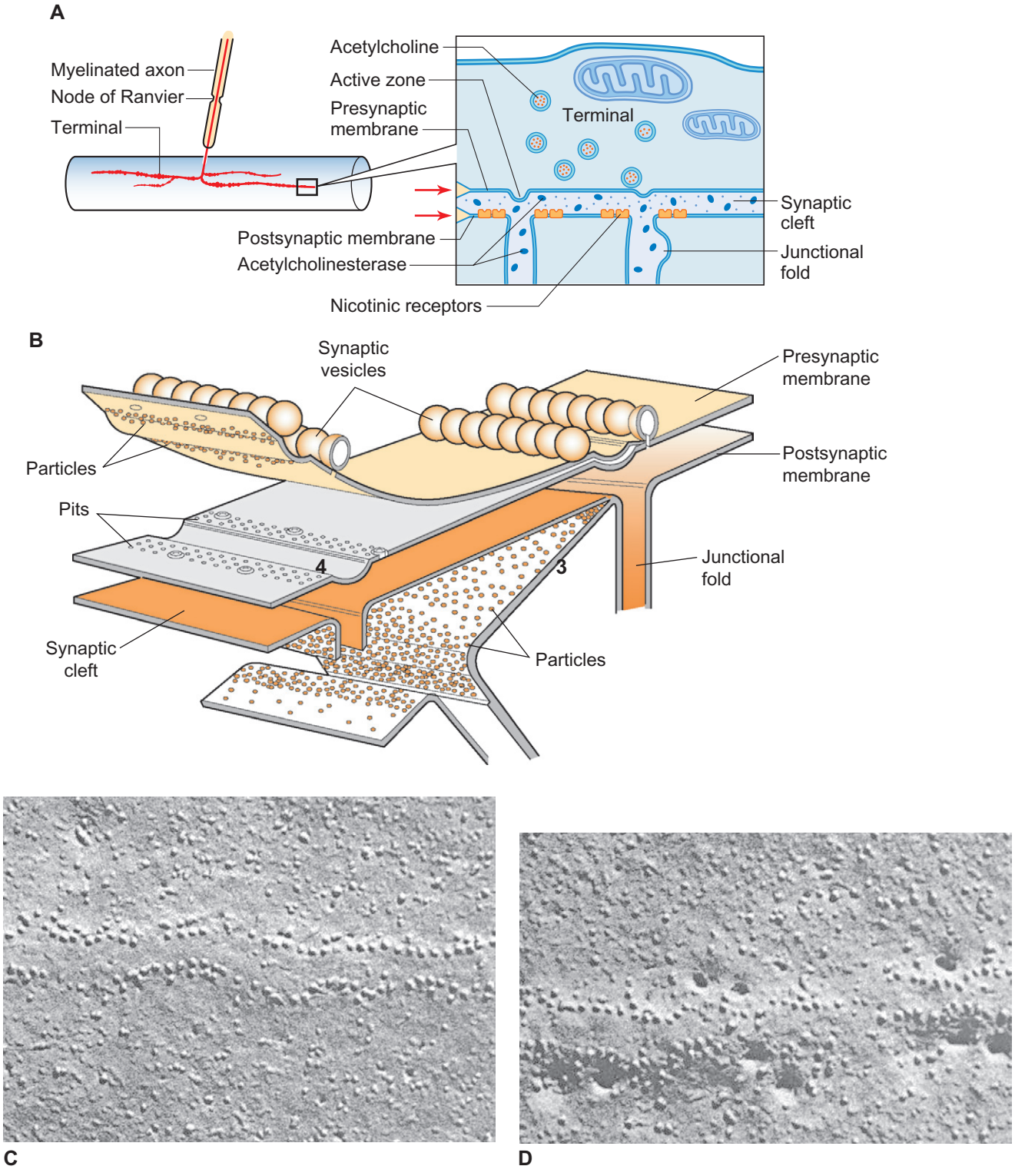


FIGURE 12-3 **Synaptic membrane structure.** (**A**) Entire frog neuromuscular junction (NMJ, **left**) and longitudinal section through a portion of the nerve terminal (**right**). *Arrows* indicate planes of cleavage during freeze-fracture. (**B**) Three-dimensional view of presynaptic and postsynaptic membranes with active zones and immediately adjacent rows of synaptic vesicles. Plasma membranes are split along planes indicated by the *arrows* in **A** to illustrate structures observed by freeze-fracture. The cytoplasmic half of the presynaptic membrane at the active zone shows on its fracture face protruding particles whose counterparts are seen as pits on the fracture face of the outer membrane leaflet. Vesicles that fuse with the presynaptic membrane give rise to characteristic protrusions and pores in the fracture faces. The fractured postsynaptic membrane in the region of the folds shows a high concentration of particles on the fracture face of the cytoplasmic leaflet; these are probably acetylcholine

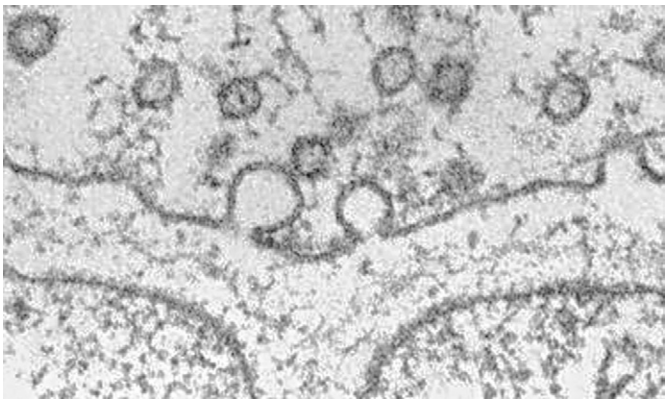


FIGURE 12-4 **High-magnification (×145,000) view of freeze-substituted neuromuscular junctions in a muscle frozen during the abnormally large burst of acetylcholine release that is provoked by a single nerve stimulus in the presence of 2 mmol/l 4-aminopyridine.** The stimulus was delivered 5.1 ms before the muscle was frozen. The section was cut unusually thin (~200 Å) to show the fine structure of the presynaptic membrane, which displayed examples of synaptic vesicles apparently caught in the act of exocytosis. In all cases, these open vesicles were found just above the mouths of the postsynaptic folds, hence at the site of the presynaptic active zones. (With permission, Heuser, 1976.)

caused by depolarization of the plasma membrane opens voltage-sensitive Ca^{2+} channels. Ca^{2+} influx and the resultant rise in the cytosolic Ca^{2+} concentration adjacent to release sites along the plasma membrane trigger exocytosis. The sites of exocytosis are closely associated with Ca^{2+} channels (Fig. 12-3C). Ca^{2+} channels may, in fact, be components of multimeric protein complexes involved in exocytosis. Intracellular $[Ca^{2+}]$ immediately adjacent to Ca^{2+} channels is probably in the range 50 to 100 μM (Simon & Llinas, 1985; Augustine et al., 1996). It is this high Ca^{2+} concentration that triggers exocytosis. Neuroendocrine cells, such as chromaffin cells from the adrenal medulla, also release hormones, such as epinephrine and opioid peptides, upon Ca^{2+} influx through membrane channels. It is thought that in this type of cell, release sites are usually not closely associated with Ca^{2+} channels and that $[Ca^{2+}]$ in the 0.5 to 10 μM range can trigger exocytosis. It should be noted that other types of cells, such as exocrine cells (for example, pancreatic acinar cells), also release stored protein by exocytosis upon a rise in cytosolic Ca^{2+}. In many cases, Ca^{2+} is released from intracellular stores by inositol trisphosphate (IP_3), which is generated by the hormonal activation of G-protein–linked receptors that activate phosphoinositide-specific phospholipase C (PLC) (Chap. 23). In this case, extracellular Ca^{2+} sustains secretion by refilling the intracellular, IP_3-sensitive Ca^{2+} stores rather than by directly triggering secretion.

Presynaptic events during synaptic transmission are rapid, dynamic and interconnected

The time between Ca^{2+} influx and exocytosis in the nerve terminal is very short. At the frog NMJ at room temperature, 0.5 to 1 ms elapses between the depolarization of the nerve terminal and the beginning of the postsynaptic response. In the squid giant synapse, recordings can be made simultaneously in the presynaptic nerve terminal and in the postsynaptic cell. Voltage-sensitive Ca^{2+} channels open toward the end of the action potential. The time between Ca^{2+} influx and the postsynaptic response is 200 μs (Fig. 12-7). Recent studies of synaptic transmission between CNS neurons using optical methods to record presynaptic events indicate a delay of only 60 μs between Ca^{2+} influx and the postsynaptic response at 38°C (Sabatini & Regehr, 1996).

The short delays between Ca^{2+} influx and exocytosis have important implications for the mechanism of fusion of synaptic vesicles (Chap. 7). In this short time, a synaptic vesicle cannot move significantly and must be already at the release site. From the diffusion constant of Ca^{2+} in squid axoplasm, one calculates that Ca^{2+} could diffuse only 850 Å, somewhat greater than the diameter of a synaptic vesicle. Therefore, in fast synapses, release sites must be close to the Ca^{2+} channels that trigger exocytosis. Vesicles are exposed to $[Ca^{2+}]$ in a concentration of a few hundred micromolar near the mouth of the channels.

The supply of synaptic vesicles in the nerve terminal is limited. With continuous stimulation of the NMJ, the number of quanta released can exceed by many fold the number immediately available in the nerve terminal. Transport of secretory vesicles from the cell body would be much too slow to maintain fast synaptic transmission in the terminal. Instead, the synaptic vesicle membrane, which fuses with the plasma membrane, is rapidly recycled via clathrin-mediated endocytosis (see Chap. 7). Hence, the vesicle membrane is a reusable container for neurotransmitter storage and exocytosis. The process of membrane recycling at the nerve terminal is closely related to the general process of endocytosis that occurs in non-neuronal cells. Strong evidence for this process came from electron micrographs of horseradish peroxidase uptake from the extracellular medium into the nerve terminal of the frog NMJ following nerve stimulation (Heuser & Reese, 1973; Ceccarelli et al., 1973). Endocytosis is dispersed along the membrane away from active zones. It was originally proposed that clathrin-coated vesicles bud from the plasma membrane, lose their triskelion clathrin coat and fuse to an intermediary endosomal compartment, from which new synaptic vesicles bud. Synaptic vesicles then take up neurotransmitter and recycle to release sites. Other studies suggest an alternative pathway that bypasses the intermediate endosomal compartment. It would allow more rapid endocytic recycling of the synaptic vesicle membrane. Clathrin-coated vesicles bud from the plasma membrane, become uncoated, take up

◀ receptors (AChRs). (Courtesy of U. J. McMahan; with permission from reference 8.) Freeze-fractured active zones from frog resting and stimulated NMJ. (**C**) The active zone is the region of presynaptic membrane surrounding double rows of intramembrane particles, which may be channels for Ca^{2+} entry that initiates transmitter release. (**D**) Holes that appear in active zones during transmitter release are openings of synaptic vesicles engaged in exocytosis. This muscle was prepared by quick-freezing, and transmitter release was augmented with 4-aminopyridine so that the morphological events, such as the opening of synaptic vesicles, could be examined at the exact moment of transmitter release evoked by a single nerve shock (×120,000). (With permission, (Heuser & Reese, 1977.)

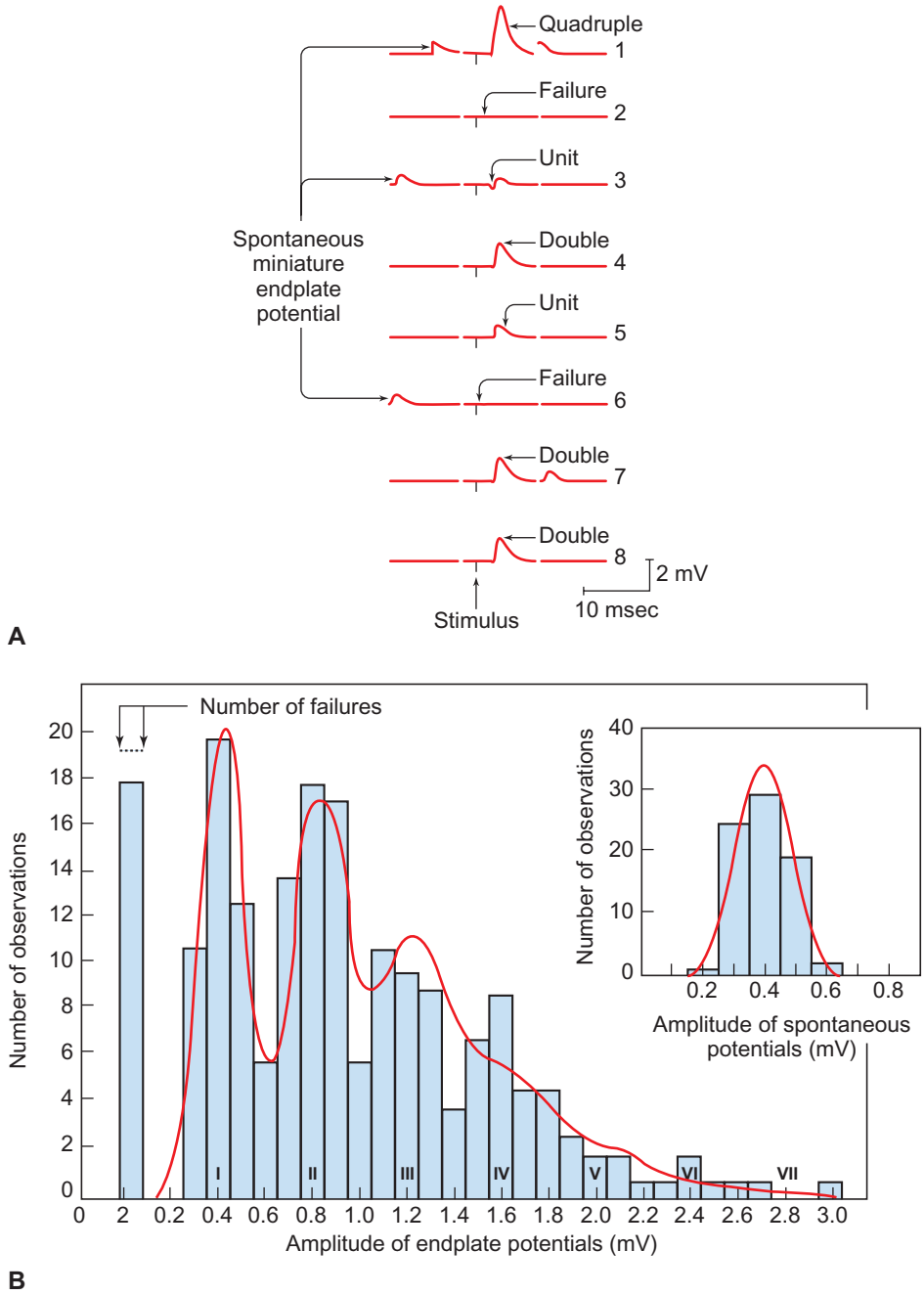


FIGURE 12-5 Comparison of the amplitudes of the spontaneous miniature endplate potentials and the evoked endplate potentials indicates that transmitter is released in quantal packages that are fixed in amplitude but variable in number. (**A**) Intracellular recording from a rat nerve–muscle synapse shows a few spontaneous miniature endplate potentials and the synaptic responses, or endplate potentials, evoked by eight consecutive stimuli to the nerve. The stimulus artifact evident in the records is produced by current flowing between the stimulating and recording electrodes in the bathing solution. In a Ca^{2+}-deficient and Mg^{2+}-rich solution designed to reduce transmitter output, the endplate potentials are small and show considerable fluctuations: two impulses produce complete failures (*2* and *6*); two produce a unit potential (*3* and *5*) and still others produce responses that are two to four times the amplitude of the unit potential. Comparison of the unit potential and the spontaneously occurring miniature endplate potential illustrates that they are the same size. (Adapted with permission from reference [12].) (**B**) Distribution of amplitudes of the spontaneous miniature endplate potentials and the evoked endplate potentials. Synaptic transmission has again been reduced, this time with only a high-Mg^{2+} solution. The histograms of the evoked endplate potential illustrate peaks that occur at 1, 2, 3 and 4 times the mean amplitude of the spontaneous potentials (0.4 mV). The distribution of the spontaneous miniature endplate potentials shown in the **inset** is fitted with a Gaussian curve. The Gaussian distribution for the spontaneous miniature potentials is used to calculate a theoretical distribution of the evoked endplate potential amplitudes, based on the Poisson equation, that predicts the number of failures, unit potentials, twin and triplet responses and so on. The fit of the data to the theoretical distribution is remarkably good (*solid line*). Thus, the actual number of failures (*dashed line* at 0 mV) was only slightly lower than the theoretically expected number of failures (*arrows above dashed line*). (Adapted with permission, Boyd & Martin, 1956.)

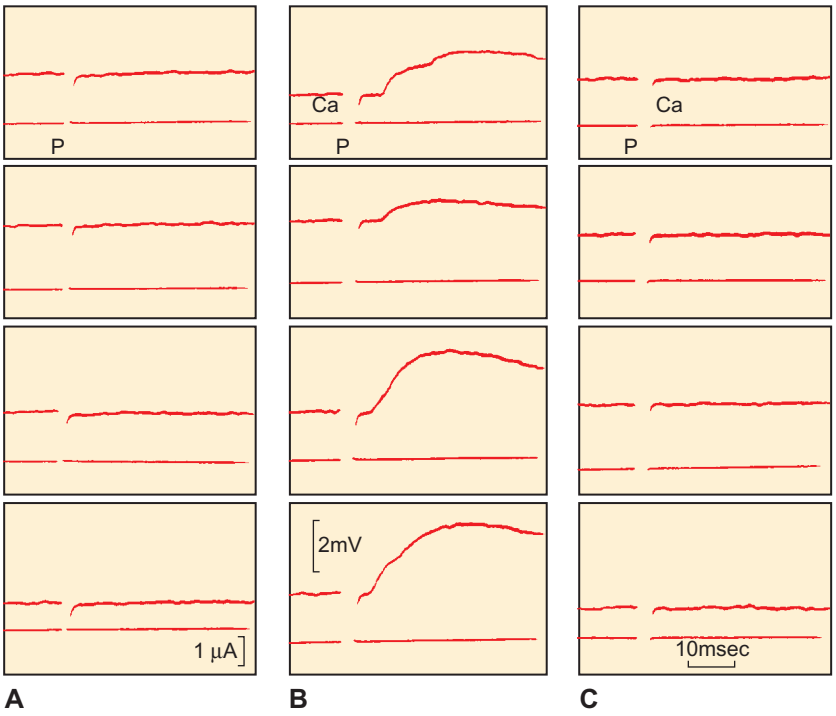


FIGURE 12-6 **Synaptic transmission requires that Ca^{2+} be present during the action potential.** The effects of iontophoretic pulses of Ca^{2+} on endplate response are shown. Depolarizing pulses (*P*) and Ca^{2+} were applied from a double-barrel micropipette to a small part of a frog sartorius neuromuscular junction. Intracellular recording was from the endplate region of the muscle fiber. **Top traces** show the postsynaptic membrane potential responses. **Bottom traces** show current pulses through the pipette. (**A**) Depolarizing pulses alone. (**B**) Short-duration, approximately 1 ms Ca^{2+} pulses applied less than 1 ms before the depolarizing pulse. (**C**) Short Ca pulses immediately following depolarizing pulses. The acetylcholinesterase inhibitor prostigmin was present to enhance the response. Temperature 3°C. Depolarization elicited endplate potentials only if the Ca^{2+} pulse preceded the depolarizing pulse (**B**). (With permission, Katz et al., 1967.)

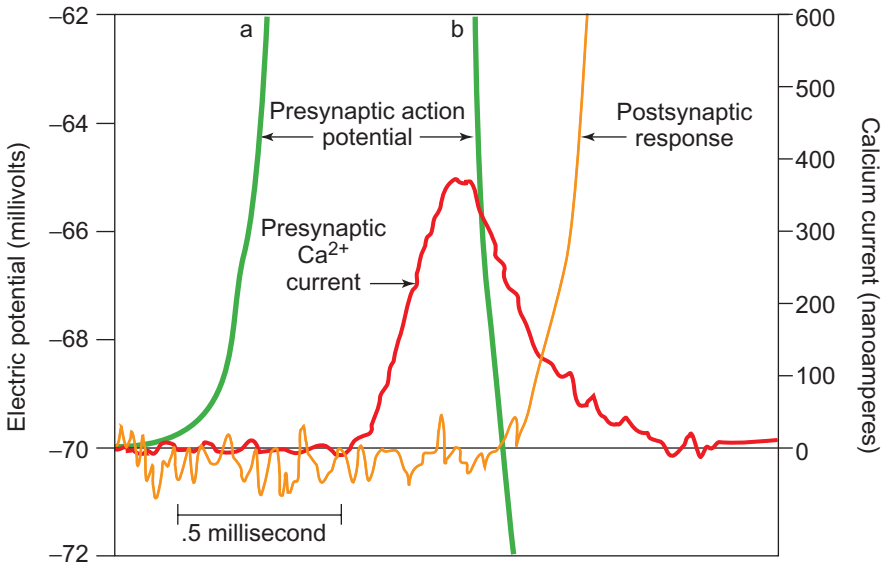


FIGURE 12-7 **The delay between Ca^{2+} influx into the nerve terminal and the postsynaptic response is brief.** The temporal relationships between the Ca^{2+} current and the action potential in the nerve terminal and the postsynaptic response in the squid giant synapse are shown. The rapid depolarization (*a*) and repolarization (*b*) phases of the action potential are drawn. A major fraction of the synaptic delay results from the slow-opening, voltage-sensitive Ca^{2+} channels. There is a further delay of approximately 200 μs between Ca^{2+} influx and the postsynaptic response. (With permission, Llinas, 1982.)

neurotransmitter and recycle to the plasma membrane. Strong stimulation of the nerve terminal may cause invaginations of the plasma membrane, from which clathrin-coated vesicles can also bud (see Chap. 2).

The development of amphipathic fluorescent dyes that label endocytic vesicles has permitted the study of endocytosis in nerve terminals in real time (Betz & Bewick, 1992; Rizzoli et al., 2003). The probe FM1–43 equilibrates between the aqueous phase and the membrane but is not membrane permeant. The plasma membrane becomes fluorescent (Fig. 12-8). Upon endocytosis, the labeled membrane is internalized. When removed from the extracellular medium, the dye is retained by the endocytic vesicles but lost from the plasma membrane. Endocytic vesicles are transformed into synaptic vesicles containing FM1–43. Importantly, recycled synaptic vesicles lose the probe upon exocytosis.

This technique has permitted the dynamics of the exocytic/endocytic cycle to be investigated. At the neuromuscular junction, a readily releasable pool (RRP) and a reserve pool of vesicles coexist, the latter being released after the RRP during high-intensity stimulation. Recycling of the fused vesicle membrane requires seconds for the RRP and approximately one minute for the reserve pool, indicating discrete endocytic pathways. A single nerve impulse releases ~0.1% of the total recycling pool. Readily releasable and reserve vesicle pools also occur in cultured hippocampal neurons (Ryan & Smith, 1995). However, in contrast to the neuromuscular junction, there is significant mixing between the pools. Endocytosis follows approximately 20 sec after exocytosis. The transformation of the endocytic vesicle into a functioning synaptic vesicle requires about 15 sec. About 0.5% of the recycling pool is released by a nerve impulse. This corresponds to approximately one vesicle per synaptic bouton.

The importance of endocytosis for the normal function of the nerve terminal is demonstrated by the *shibire* mutant of *Drosophila*. When these mutant flies, bearing a temperature-sensitive allele, are exposed to high temperature, they become paralyzed within 1 min but rapidly recover when returned

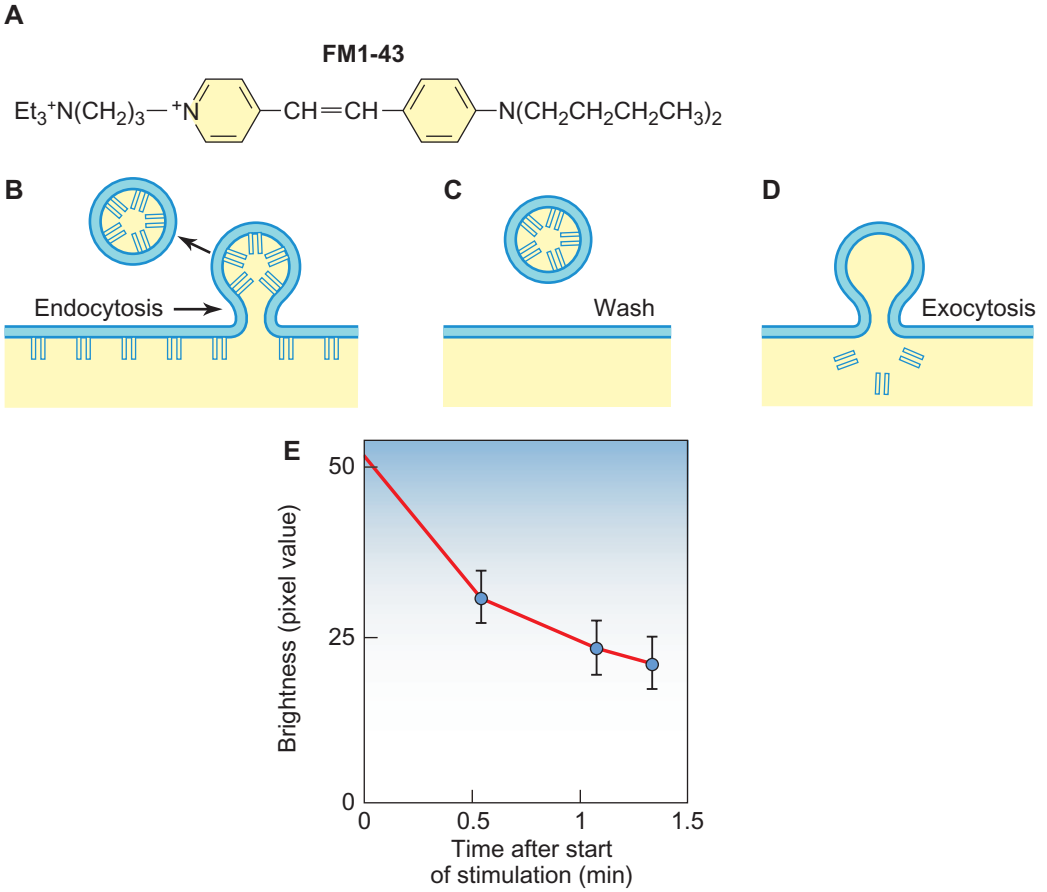


FIGURE 12-8 **The probe FM1-43 was used to visualize endocytosis and exocytosis at the neuromuscular junction (NMJ).** **(A)** Structure of the amphipathic membrane probe FM1-43. **(B)** Labeling of the plasmalemma by FM1-43 in the extracellular medium. The amphipathic probe is present during electrical stimulation of the NMJ. Note that membrane originating from synaptic vesicles that have undergone exocytosis is labeled. (**C**) A brief wash of the NMJ after electrical stimulation removes FM1-43 from the plasma membrane but not from intracellular endocytic vesicles that had formed following exocytosis in the presence of FM1-43. (**D**, **E**) A second round of exocytosis stimulated by exocytosis in the absence of extracellular FM1-43 results in loss of the fluorescent probe from newly formed synaptic vesicles that have undergone exocytosis. In **E**, the fluorescence intensity of vesicle populations at the NMJ was followed over time during a 10 Hz stimulation. Note the decline of fluorescence as FM1-43-labeled vesicles undergo exocytosis and release the probe into the extracellular medium. (Betz & Bewick, 1992).

to the permissive temperature. Electron microscopy demonstrates that the paralysis results from a block of synaptic vesicle endocytosis at the NMJ. The *shibire* allele encodes dynamin, a GTPase that is essential for the fission of the endocytic bud from the plasma membrane (Chen et al., 1991; van der Bliek & Meyerowitz, 1991).

Because fast synaptic transmission involves recycling vesicles, the neurotransmitter must be replenished locally

Thus, fast synaptic transmission uses neurotransmitters such as ACh, glutamate, GABA, glycine, dopamine (DA) and NE, all of which can be synthesized within the nerve terminal or transported rapidly across the nerve terminal plasma membrane. In contrast, proteins are inserted into secretory granules in the cell body. The secretory granules must then be transported by fast axonal transport into the nerve terminal, a process that can take many hours or days depending on the distance of the nerve terminal from the soma (see Chap. 8). Nerve terminals that are specialized for fast synaptic transmission often have peptidergic granules as well as the recycling vesicles of fast synaptic transmission. For example, nerve terminals can contain VIP as well as ACh, enkephalin as well as NE and substance P as well as 5-HT. The peptidergic granules are usually far less numerous than the smaller vesicles involved in fast exocytosis and are not localized at active zones (Fig. 12-1). The exocytosis of protein-containing granules in nerve terminals may be closely related to exocytosis of protein-containing granules in endocrine and exocrine cells. While a single nerve impulse will release vesicles at active zones, exocytosis of peptidergic granules in nerve terminals can require multiple or high-frequency stimulations. This may reflect the need for sustained elevations of Ca^{2+} that extend into the interior of the nerve terminal. Peptides and proteins released from nerves may have slower and longer-lasting effects on postsynaptic cells than fast neurotransmitters and can modulate the response to fast neurotransmitters.

Discrete steps in the regulated secretory pathway can be defined in neuroendocrine cells

The rapid presynaptic events of synaptic transmission produce closely coordinated exocytosis and endocytosis. Insights into the steps involved in the exocytotic limb of the pathway have come from the studies of kinetics of secretion of protein-containing granules from adrenal chromaffin cells and PC12 cells. Adrenal chromaffin cells are excitable and contain a large number of secretory or chromaffin granules. In bovine chromaffin cells, a neuronal-type nAChR and voltage-sensitive Ca^{2+} channels permit Ca^{2+} entry, which stimulates secretion. The intracellular milieu of chromaffin cells can be directly controlled by extracellular solutions in cells with plasma membranes rendered leaky by the detergent digitonin (Holz et al., 1992), or by mechanically disrupting the plasma membrane by passage of the cells through a steel cylinder partially blocked by a precision steel bearing (Martin & Walent, 1989). PC12 cells contain far fewer granules than adrenal chromaffin cells, and many are closely associated with the plasma membrane. An analysis of the effects of Ca^{2+}, ATP and temperature suggests that ATP hydrolysis occurs before Ca^{2+} is able to cause secretory granules to secrete. Two distinct Ca^{2+}-dependent steps have been identified. One triggers exocytosis with maximal effects at 100 to 300μM Ca^{2+}, whereas the other enhances the ability of ATP to prime secretion, with maximal effects at approximately 1μM Ca^{2+}. Electrophysiological studies have identified additional steps associated with the triggering of exocytosis that may reflect the dynamics of interrelated pools of granules (Rettig & Neher, 2002).

What is the function of ATP in secretion? While protein phosphorylation can modulate the secretory response, there is compelling evidence that the effect of ATP in priming involves other processes. ATP is necessary for the function of *N*-ethylmaleimide-sensitive factor (NSF). This protein is an ATPase that acts as a molecular chaperone to dissociate complexes of the SNARE proteins VAMP (synaptobrevin), syntaxin and SNAP-25 (see Chap. 7). This may permit their subsequent reassociation as part of the exocytotic response. Another function of ATP in priming exocytosis is the maintenance of the polyphosphoinositides, phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP), by phosphorylation of lipid precursors via phosphatidylinositol 4-kinase and PIP kinase (Eberhard et al., 1990; Hay et al., 1995). Interestingly, phosphatidylinositol 4-kinase is an integral membrane protein of chromaffin granules and synaptic vesicles. The polyphosphoinositides appear to function in the priming step not as precursors for the formation of IP_3 and diacylglycerol (DAG) but, rather, in another capacity (see Chap. 23). PIP_2 binds specifically to the vesicle or granule proteins synaptotagmin and rabphilin3. PIP_2 also regulates numerous proteins that control the cytoskeleton, such as profilin, gelsolin, scinderin and myosin I. Therefore, the polyphosphoinositides on the secretory granule membrane may coordinate the function in secretion of several secretory granule proteins and may modulate dynamic changes in the cytoskeletal network that are important for exocytosis. PIP_2 also plays an essential role in endocytosis in presynaptic nerve terminals through the regulation of proteins involved with coating and uncoating of endocytic vesicles and through the regulation of the actin cytoskeleton.

CELLULAR SIGNALING MECHANISMS

Background

Largely as a result of the studies of Langley, the possibility that biological tissues possess receptor molecules that are specific for each neurotransmitter was first entertained in the early 1900s. Langley noted the high degree of specificity and potency with which some agents elicit a biological response and postulated the existence of "receptor" or "acceptor" molecules. This concept has subsequently been fully validated. Many receptors have been isolated and purified biochemically, and many have also been cloned and sequenced. In several cases, the activity of purified receptors has been reconstituted in artificial systems.

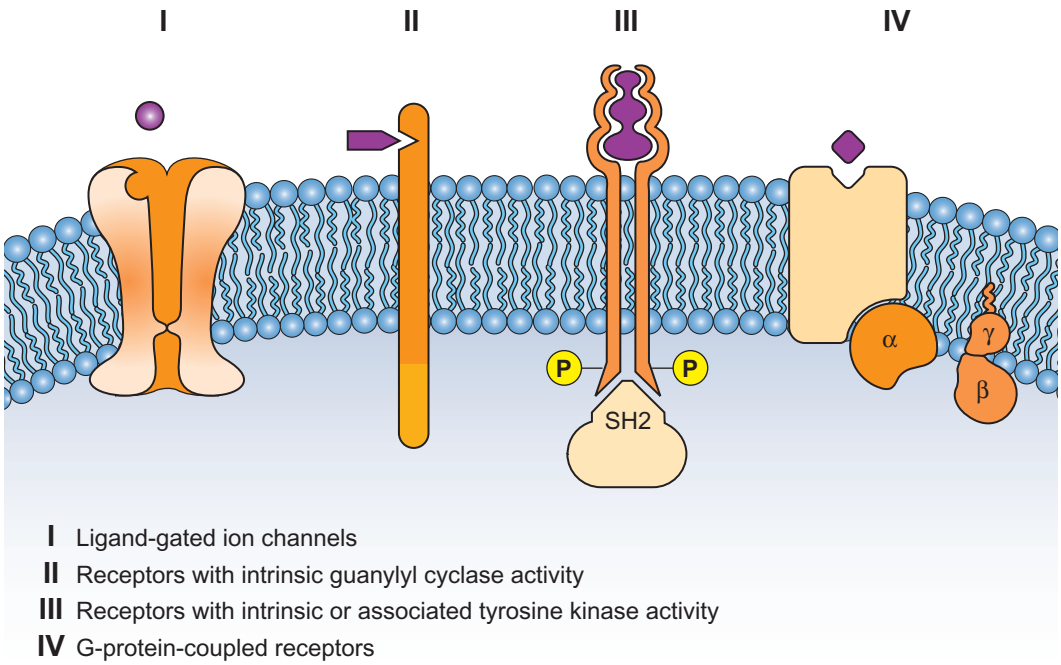


FIGURE 12-9 **Cell-surface receptors utilize four distinct molecular mechanisms for transmembrane signaling.** *I*. Ligand-gated ion channels. *II*. Receptors which possess intrinsic guanylyl cyclase activity. *III*. Receptors with intrinsic or associated tyrosine kinase activity. *IV*. G-protein-coupled receptors, which are linked to the opening/closing of ion channels, modulation of adenylyl cyclase and phosphoinositide-specific phospholipase C activities. *SH2*, Src homology 2 domain.

Three phases of receptor-mediated signaling can be identified

The first is the binding component in which an extracellular ligand, usually a polar molecule, forms a complex with a cell-surface receptor, which can be localized on either a pre- or a postsynaptic membrane. The second phase is that in which the activated receptor–ligand complex elicits an increase in either the formation of second messengers, the opening or closing of membrane ion channels or the recruitment of cytoplasmic proteins to the membrane in the proximity to the receptor–ligand complex. The third phase typically involves the activation of cytosolic enzymes, typically protein kinases or phosphatases, which mediate the biological response. Thus, the initial interaction of a ligand with a receptor results in amplification of the signal by means of a cascade of responses.

Several major molecular mechanisms that link agonist occupancy of cell-surface receptors to functional responses have been identified

Traditionally, receptors have been classified according to the mediator to which they respond. The first example, proposed by Sir Henry Dale in 1914, was that the neurotransmitter ACh can interact with two types of AChRs, termed either nicotinic or muscarinic AChRs, based on the similarity of action of ACh to the plant alkaloids nicotine and muscarine. Similarly, Ahlquist proposed the division of adrenergic receptors into α and β subtypes, based on the potency of a series of natural and synthetic agonists to elicit a biological response. Although the pharmacological characterization of receptors provided a useful starting point, it rapidly became apparent that extensive subclassification of receptors would be required. For example, it is now recognized that there are both neural and non-neural forms of the nAChR, which can be distinguished pharmacologically and biochemically (Ch.13). In addition, multiple adrenergic receptor subtypes, including α_1, α_2, β_1, β_2 and β_3, can be distinguished based on the ability of selective agonists and antagonists to bind to them (Ch. 14).

Receptors can also be grouped according to the type of primary effector to which they couple. This classification leads to four major categories of receptors (Fig. 12-9 and Table 12-1).

First group

The first group is composed of receptors that possess intrinsic ion channels composed of multiple subunits. Upon the binding of an agonist to these ligand-gated ion channels, the receptors undergo a conformational change that facilitates opening of the intrinsic ion channel. The permeability to specific ions is a characteristic of the receptor; for example, both the neuronal nicotinic cholinergic receptors (nAChR) and N-methyl D-aspartate (NMDA) receptors are selectively permeable to Na^+ and Ca^{2+} ions, whereas $GABA_A$ and glycine receptors are primarily permeable to Cl^- ions. As a result of

TABLE 12-1 Examples of Cell-Surface Receptors that Operate Via Distinct Signaling Mechanisms

	Ligand or stimulus	Receptor(s) and subtypes	Chapter
Ligand-gated ion channels	Acetylcholine	Nicotinic cholinergic (muscle [$\alpha\beta\gamma\delta\epsilon$] and neuronal [$\alpha$ or $\alpha\beta$] subtypes)	13
	ATP	P_{2X1-7}	19
	GABA	$GABA_A$	18
	Glutamate	NMDA (GluN1, GluN2A-D, GluN3A,B), AMPA (GluA1-4) and Kainate (GluK1-3, 4-5)	17
	Glycine	GlyR	Box
	Inositol 1,4,5 trisphosphate	IP_3-R1, IP_3-R2, IP_3-R3	23
	Serotonin	$5HT_3$	15
Receptors with intrinsic guanylyl cyclase activity	Brain naturetic peptide	GC-B	22
Receptors with intrinsic or associated tyrosine kinase activity	Brain-derived neurotrophic factor	TrkB	29
	Epidermal growth factor	EGFR	29
	Fibroblast growth factors	FGFR1- FGFR4	29
	Insulin-like growth factor 1	IGFR-1	29
	Nerve growth factor	Trk A	29
	Neuregulins	ErbB2, ErbB3 and ErbB4	29
	Neurotrophin 3	Trk C	29
	Neurotrophin 4/5	Trk B	29
	Platelet-derived growth factor	PDGFR α and β	29
	Ciliary neurotrophic factor	gp130 + CNTFRα and LIFRβ	29
	Interleukin-6	2 x gp130 + IL6Rα	29
	Leukemia inhibitory factor	gp130 + LIFRβ	29
G-protein-coupled receptors	Acetylcholine	M1, M3 and M5; M2 and M4	13
	Adenosine	A_1, A_{2A}, A_{2B} and A_3	19
	ATP	$P_{2Y1,2,4,6,11-14}$	19
	Dopamine	D1,D5; D2-D4	14
	Endocannabinoids	CB1, CB2	61
	GABA	$GABA_B$	18
	Glutamate	mGlu1,5; mGlu2,3; mGlu4,6,7,8	17
	Histamine	H_1,H_2; H_3 and H_4	16
	Light	Rhodopsin	51
	Norepinephrine	α_{1A} α_{1B} α_{1C}, α_{2A} α_{2B} α_{2C}, β_1,β_2,β_3	14
	Odorants	ORs, V1Rs, V2Rs	52
	Opioids	μ, δ and κ	20
	Prostaglandins	DP, EP_{1-4}, FP, IP	36
	Serotonin	5-HT1, 5-HT2, 5-HT4, 5-ht5A, 5-ht5B, 5-ht6, 5-HT7	15
	Substance P	NK1	60
	Taste (bitter, sweet and umami)	T1R-T3R	52
	Thromboxane	TP	36

the changes in ion conductance, the membrane potential may become either depolarized, as occurs for nAChRs or NMDA receptors, or hyperpolarized, as observed for $GABA_A$ or glycine receptors. Receptors in this category include those that are activated by synaptically released neurotransmitter and occur on the cell surface. The initial responses to these cell-surface receptors are extremely rapid, occurring in milliseconds, and do not require either the subsequent generation of second-messenger molecules or protein phosphorylation events. Thus, ligand-gated ion channels mediate "fast synaptic" transmission events in the CNS. However, some ligand-gated ion channel receptors (e.g., NMDA and $GABA_A$) are also found at extrasynaptic locations. Not all ligand-gated ion channels are present on the cell surface. For example, the intracellular ligand-gated receptor for IP_3 is present in the smooth endoplasmic reticulum (see Chap. 23).

Second group

Receptors in the second group possess intrinsic guanylyl cyclase activity and generate cyclic GMP (cGMP) upon activation of a receptor; for example, the brain natriuretic peptide receptor. These receptors consist of an extracellular binding domain, a single transmembrane-spanning domain (TMD), a protein kinase–like domain and a guanylyl cyclase catalytic domain (see Chap. 22). Ligand binding results in a conformational change in the receptor and activation of the guanylyl cyclase catalytic region. Membrane-bound guanylyl cyclase activity does not require Ca^{2+} and can be modulated by ligand addition to cell-free preparations. A different, cytoplasmic form of guanylyl cyclase is activated by micromolar concentrations of Ca^{2+}. Receptors with intrinsic guanylyl cyclase activity are often very highly phosphorylated in the absence of agonist and rapidly undergo dephosphorylation upon activation.

Third group

Receptors in the third group possess intrinsic receptor tyrosine kinase (RTK) activity themselves or are closely associated with cytoplasmic tyrosine kinases (RATK). RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), are found in all multicellular eukaryotic organisms and are involved in the regulation of cellular growth and differentiation (see Chs. 26 and 29). Structurally, RTKs possess an extracellular ligand-binding domain, a single TMD and an intracellular catalytic kinase domain. Three distinct events underlie signal transduction at RTKs. Initially, upon ligand binding to an RTK, the receptor undergoes a dimerization that results in the juxtaposition of the two cytoplasmic domains. Contact between these domains is thought to result in a stimulation of catalytic activity, which in turn results in an intermolecular autophosphorylation of tyrosine residues both within and outside of the kinase domain. The significance of the phosphorylation of tyrosine residues lies in the subsequent ability of the RTK to recruit cytoplasmic proteins that possess Src homology 2 (SH2) domains. These regions of the molecule are 60 to 100 amino acids in length, are globular in structure, protrude from the surface of the protein and permit high-affinity protein–protein interactions to occur. In the case of the SH2 domain, a key arginine residue buried deep in a specific binding pocket interacts with the phosphate group of the tyrosine residue. The presence of an SH2 domain can increase the affinity of a peptide for a phosphorylated tyrosine residue by 1,000 fold. Once autophosphorylated, RTKs can recruit a number of cytoplasmic proteins and initiate a series of reactions involving protein–protein interactions. The best-studied pathway of this type is the mitogen-activated protein kinase (MAPK) pathway. RTKs, via the recruitment of an adaptor protein complex such as growth factor receptor–binding protein 2 (Grb2)/son of sevenless (SOS) or SHC (see Chap. 29) can activate Ras, a low-molecular-weight monomeric G protein. The role of Ras is to recruit and activate Raf (MAPKKK), a serine/threonine kinase, which in turn activates MEK (MAPKK), a dual-specificity tyrosine/threonine kinase. MEK subsequently activates MAPK, also known as ERK or extracellular signal–regulated kinase, which is a serine/threonine kinase with multiple substrates. ERK can enter the nucleus and regulate gene transcription by phosphorylating nuclear proteins (see Chap. 25). RATKs, such as those for the neurotrophic cytokines (leukemia inhibitory factor, interleukin-6 or ciliary neurotrophic factor) do not possess intrinsic tyrosine kinase activity themselves, but upon activation, they undergo dimerization and are then able to recruit cytoplasmic tyrosine kinases (such as Janus kinase). The latter then phosphorylate the RATK on tyrosine residues (in addition to being tyrosine phosphorylated themselves) and facilitate protein–protein interactions, as observed for RTKs.

Fourth group

The fourth group of receptors involves G proteins. Numerically, more diverse types of receptors have been demonstrated to operate via an intervening G protein than by any other mechanism. These G protein–coupled receptors (GPCRs), which have a characteristic seven TMD structure, can be further divided into four functional categories. Some GPCRs, such as $GABA_B$, α_2-adrenergic, D2-dopaminergic or M2 muscarinic (mAChR), regulate the changes in K^+ conductance independently of second-messenger production (see Chap. 21). A second group of GPCRs is linked to the modulation of adenylyl cyclase activity. This regulation may be either positive, as in the case of activation of the β_2-adrenergic receptor, or negative, as occurs following activation of the α_2-adrenergic receptor. Changes in the concentrations of cAMP regulate the activity of protein kinase A (PKA) (Chap. 22). Note that, on occasions, the same GPCR may activate more than one effector mechanism. Thus M2 mAChRs regulate both K^+ conductance and adenylyl cyclase activity in the heart. A third group of GPCRs is linked to the activation of phosphoinositide-specific phospholipase C (PLC) with the attendant breakdown of PIP_2 and formation of IP_3 and DAG (see Chap. 23). These receptors are linked to changes in Ca^{2+} homeostasis and protein phosphorylation via the action of protein kinase C (PKC). Other effector enzymes that may be regulated by IP_3-linked GPCRs include phospholipases A_2 and D. A fourth, and unique, mechanism for the activation of a GPCR is that utilized by the visual pigment rhodopsin, which structurally is a prototypical GPCR. However, in this case it is light, rather than a chemical

stimulus, that triggers the activation of rhodopsin. Upon the absorption of light, the chromophore, 11-*cis*-retinal, which is covalently bound to the opsin protein, undergoes isomerization to all *trans*-retinal. Photoactivated rhodopsin activates transducin, a G-protein, which is coupled to cGMP phosphodiesterase with a concomitant increased rate in the hydrolysis of cGMP to GMP. The loss of cGMP results in the closure of plasma membrane cation channels in the rod outer segments (see Chap. 51).

Cross-talk can occur between intracellular signaling pathways

Most cells possess receptors that operate through each of these four distinct effector mechanisms. Receptors within each class may be subject to further regulation during persistent agonist occupancy. One way that this can occur is via phosphorylation of the receptor, which results in its uncoupling from the effector enzyme, as has been demonstrated for the β-adrenergic receptor (see Chap. 14). This has been called *homologous regulation*. In addition, signaling pathways do not operate in isolation but may regulate and be regulated by one another, which has been termed *heterologous regulation*. In this way, the output of the cell is fine-tuned via subtle modulation of the relevant intracellular signaling mechanisms. There are numerous examples in which the activity of one receptor can regulate, either positively or negatively, the activity of a second; for example, increases in cAMP mediated by PKA can depress or potentiate PLC activity. Conversely, activation of PLC can result in modulation of the activity of other pathways via the activation of PKC. An additional complexity is that the same ligand may activate multiple pathways in a given tissue. For example, NE can activate β_2-adrenergic receptors, which increase adenylyl cyclase activity; α_2-adrenergic receptors, which are coupled to an inhibition of adenylyl cyclase activity; and α_1-adrenergic receptors, which are linked to the activation of PLC. Similarly, ACh can activate muscarinic cholinergic receptors, which inhibit adenylyl cyclase activity, increase PLC activity or facilitate the opening of K^+ channels. In addition, ACh can directly activate nAChRs, which are linked to changes in Na^+ and Ca^{2+} permeability.

Thus, although individual signaling mechanisms are most frequently studied in isolation, their activity *in vivo* is likely to be highly regulated by other signal-transduction events.

A further consideration is that receptors that primarily activate one pathway may, on occasion, activate a second pathway. An example is the ability of GPCRs, such as α_2-adrenergic receptors or mAChRs, to activate the MAPK cascade. Activation of adenylyl cyclase-linked receptors results in the release of G-protein βγ subunits, which, via an intermediary protein tyrosine kinase, stimulates phosphorylation of the adaptor protein SHC. This in turn recruits the Grb2–SOS complex and activates the MAPK pathway.

Activation of PLC-linked receptors, such as the mAChR, results in increased PKC activity. Since the addition of phorbol esters, which are PKC agonists (Chap. 23), results in phosphorylation of Raf, this mechanism represents an alternative means whereby PLC-coupled receptors can activate MAPK.

Signaling molecules can activate gene transcription

In addition to their ability to elicit acute effects within cells, second-messenger molecules, such as cyclic AMP (cAMP), Ca^{2+} and DAG, can regulate gene transcription. The transcription factors cAMP response element–binding (CREB) protein, fos and jun, which respond to these signaling molecules, are members of the amphipathic helix family of proteins, and each contain a characteristic "leucine zipper" that mediates dimerization (Chap. 27). The best studied of these transcription factors is CREB, which becomes phosphorylated on Ser^{133} in response to increases in the intracellular concentration of cAMP and subsequent activation of PKA. CREB can also be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase IV. In its phosphorylated form, CREB binds to an eight-base *cis*-regulatory sequence called cAMP response element (CRE) and stimulates transcription. CREB has been implicated in long-term potentiation and memory (see Chap. 56). Increased concentrations of cytoplasmic Ca^{2+} and DAG activate PKC, which can also activate gene transcription via fos and jun. Activation of the MAPK pathway also leads to increased expression of jun and fos. Because increases in mRNA for fos and jun are observed very rapidly following a variety of stimuli, they have been termed *immediate early genes*. The fos–jun heterodimers, termed AP-1, bind to a seven-base-pair *cis*-regulatory element called tetradecanoyl-phorbol acetate (TPA) response element (TRE). The latter has been demonstrated to control the transcription of several genes coding for neuropeptide modulators. Thus, despite major differences in the initial molecular mechanisms that underlie activation of RTKs, RATKs or receptors coupled to either adenylyl cyclase or PLC, these diverse groups of receptors share at least one common end point, that of gene regulation.

Nitric oxide acts as an intercellular signaling molecule in the central nervous system

Because cells rarely act in isolation, a signaling event in one cell may have a significant impact on the activity of neighboring cells. Initial evidence for intercellular, nonsynaptic signaling was obtained from experiments with blood vessels, in which it was observed that the addition of ACh increased cGMP concentrations and resulted in vasodilation and relaxation of the vascular smooth muscle. The increases in cGMP concentrations were dependent on the presence of Ca^{2+} and could not be demonstrated in tissue homogenates. A key observation was that removal of the endothelium abolished the relaxing effect of ACh, indicating the existence of an *endothelium-derived relaxing factor* (EDRF). Subsequently, it was discovered that EDRF was a short-lived gaseous molecule, nitric oxide (NO). NO is synthesized from arginine via the Ca^{2+} activation of nitric oxide synthase (NOS), an enzyme that requires NADPH as coenzyme and tetrahydrobiopterin as cofactor. Three distinct forms of NOS have been identified. An inducible form (iNOS), which is present in glia; a neuronal form (nNOS), which is widespread in distribution throughout the CNS; and an endothelial enzyme (eNOS) (Brendt & Snyder, 1994). The activity of both nNOS and eNOS is Ca^{2+}/calmodulin dependent, whereas that of iNOS is Ca^{2+} insensitive.

GLYCINERGIC NEUROTRANSMISSION AND NEUROLOGIC DISEASE

George J. Siegel

Introduction

It was first proposed in 1965 that glycine acts as a neurotransmitter in mammalian spinal cord (Aprison & Werman, 1965; Davidoff, et al., 1967), and since then it has been demonstrated that glycine meets all of the criteria for that designation. Glycine is widely recognized as a major inhibitory neurotransmitter in the vertebrate CNS, especially the spinal cord and brainstem, where it is crucial for the regulation of motor neuron activity (Lynch, 2009). In addition, glycinergic interneurons are found in the hippocampus, retina, auditory system and other areas involved in the processing of sensory information. γ-Aminobutyric acid (GABA) and glycine are the two major inhibitory transmitters in the CNS and both are fast acting on ligand-gated chloride ion channels. Like GABA, glycine inhibits neuronal firing by gating Cl channels but with a characteristically different pharmacology (see Ch.18) (Dresbach et al., 2008).

Biochemistry and Transport of Glycine

The immediate precursor of glycine is serine

Serine is converted to glycine by the activity of the enzyme serine hydroxymethyltransferase (SHMT) (see Fig. 42-3). Glycine is degraded intracellularly by the glycine cleavage system (see Fig. 42-3), a multienzyme complex composed of four different proteins, which, in the CNS, appears to be primarily localized in astrocytes. Mutations in the glycine cleavage system cause nonketotic hyperglycinemia, a disease characterized by severe mental retardation ('glycine encephalopathy') (details discussed in Ch. 42).

Glycine is concentrated from the cytoplasm into synaptic vesicles by the H^+-dependent vesicular transporter (VIAAT or vGAT)

VIAAT is the vesicular inhibitory amino acid antiporter that also concentrates GABA into vesicles (see Chap. 3). As is the case for GABA, Ca^2-dependent release of glycine and specific postsynaptic glycine receptors have both been rigorously demonstrated. The postsynaptic action of glycine is terminated by its reuptake via high-affinity plasmalemmal transporter systems located in glycinergic nerve terminals and glial cells. Molecular cloning has identified two glycine transporter genes, glyT1 and glyT2, which are members of the Na,Cl-dependent transporter superfamily [see in Chap. 3]. GlyT1 is abundantly expressed in astrocytes throughout the CNS, whereas GlyT2 is highly localized in glycine-releasing nerve terminals of spinal cord and brain stem. Both glycine transporters differ in their transport stoichiometries and substrate affinities and appear to have different roles at glycinergic synapses [Eulenburg & Gomeza, 2010]. GlyT1 catalyzes the removal of glycine from postsynaptic glycine receptors, whereas GlyT2 is essential for replenishing the presynaptic pool of glycine from which synaptic vesicles are reloaded with neurotransmitter. GlyT1 may, in addition, regulate glycine levels at excitatory NMDA receptors (see below), and selective GlyT1 inhibitors may therefore prove useful in the treatment of diseases associated with impaired glutamatergic transmission, such as schizophrenia [see Chs. 17 and 58].

Postsynaptic Effects and Pharmacology

A number of amino acids can activate, to varying degrees, the inhibitory glycine receptor

The amino acids that can activate the glycine receptor (GlyR) include β-alanine, taurine, L-alanine, L-serine and proline, but the receptor's greatest affinity is for glycine. GABA is largely inactive at this receptor. There are only a few known high-affinity antagonists of the inhibitory glycine receptors, including some plant compounds. The latter include the plant alkaloid strychnine, which is highly selective for the glycine receptor, and the amidine steroid RU 5135, which is less selective. Both compounds bind to the glycine receptor with nanomolar affinities. In addition, other low-affinity antagonists exist. The binding sites for glycine and competitive antagonists like strychnine are overlapping but not identical. Current findings indicate that up to three molecules of glycine are required to activate the glycine receptor. This high cooperativity may reflect the low binding energy of a ligand as small as glycine.

As for the $GABA_A$ receptor, positive modulators that enhance glycine receptor activity have been identified. These include alcohols, neurosteroids, tropeines and the divalent metal ion Zn^2, which is highly enriched in some types of excitatory neuron. Zn^2 released from such neurons may potentiate glycine receptors at neighboring inhibitory synapses and thus facilitate inhibition following strong excitation (Lynch, 2009).

Glycine is inhibitory on ligand-gated, strychnine-sensitive Cl^- channel receptors but excitatory on N-methyl-D-aspartate receptors

Although acting as a classical neurotransmitter at inhibitory ion channel receptors, glycine is also an activating ligand at a class of excitatory ion channel receptors, the N-methyl-D-aspartate (NMDA) receptors (see Ch. 17). Several investigators have shown that glycine is an essential coagonist with glutamate at the NMDA receptor and is required for channel activation in addition to glutamate. Glycine is normally present in the extracellular space at the required concentrations. Regulation of NMDA receptor activity via the release and/or reuptake of glycine may be important for allowing cross-talk between inhibitory and excitatory synapses in different regions of the CNS, including the hippocampus and spinal cord (Ahmadi et al., 2003; Thompson et al., 2010).

It appears curious that common amino acids like glycine, GABA and glutamate, which have other roles in metabolism, are employed as signaling molecules in the nervous system and that glycine is utilized as both an inhibitory and an excitatory neurotransmitter. However, sequence analysis indicates that receptors for signaling molecules evolved from prokaryotic proteins

GLYCINERGIC NEUROTRANSMISSION AND NEUROLOGIC DISEASE (*cont'd*)

utilized for binding and recognition of nutrients in the environment, including glycine, glutamate and GABA. GABA, a carbon- and nitrogen-storage molecule in plants and algae, appears to act as a colony-stimulating factor for abalone, an invertebrate mollusk. Thus, nutrient amino acids may have functioned as informational molecules early in evolution.

Structure and Function of Glycine Receptors

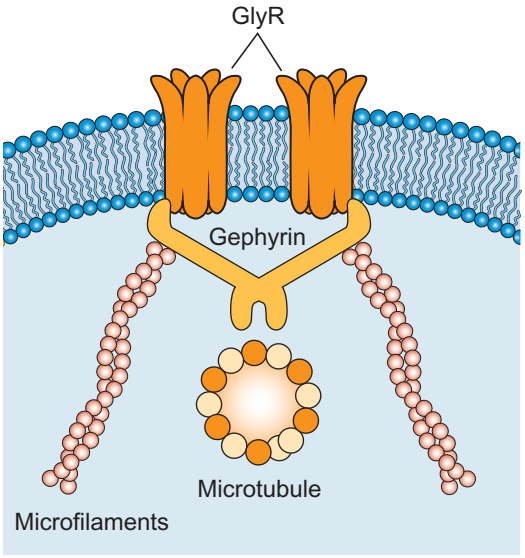
The glycine receptor belongs to the same cys-loop superfamily as does the $GABA_A$ receptor

This superfamily of fast, ligand-gated ion channel receptors includes also the nicotinic acetylcholine- and the serotonergic 5-HT_3 receptors (see details in Chaps. 13 and 15) (Absalom, et al., 2009). The transmitter binding site and the ion channel are on the same polypeptide subunit such that the conformational changes induced by the ligand binding directly affect the ion channel opening. The native glycine receptor is a macromolecular complex of about 250 kDa composed of a combination of homologous polypeptides identified as α-subunit (48 kDa) and β -subunit (58 kDa), which form a quasi-symmetrical pentameric arrangement around a central ion pore (Absalom et al., 2009; Grudzinska et al., 2005). There is approximately 50% amino acid sequence identity between the α- and β- subunits and a comparatively high homology to $GABA_A$ receptor proteins. Four α subunits (α 1, α 2, α 3, α 4) sharing >90% amino acid sequence homology and several splice variants have been cloned. Photoaffinity labeling of the glycine receptor has indicated that binding sites for glycine and strychnine are found on the α -subunit. The α -and β -subunits span the postsynaptic membrane and are glycosylated. Like the $GABA_A$ receptor and nicotinic acetylcholine receptor subunits, glycine receptor subunits each have four predicted hydrophobic segments, M1–M4, which may span the lipid bilayer as α -helices (see Fig. 18-2). Each GlyR subunit is composed of a large extracellular N-terminal domain containing the ligand-binding sites and the Cys-loop. This connects to the bundle of four alpha-helical transmembrane segments that have a large intracellular segment between M3 and M4 and a short extracellular C-terminus. The M2 domains of each of the subunits form an amphipathic lining of the central water-filled pore. The ligand-binding domain consists mainly of β-sheets forming a pocket-like structure at the subunit interface accessible from the extracellular face (Absalom et al., 2009; Dresbach et al., 2008; Lynch, 2009).

Gephyrin and collybistin are accessory proteins critical to the localization and clustering of GlyRs

Unlike the α– and β-subunits, gephyrin is a highly hydrophilic polypeptide that is cytoplasmically localized at postsynaptic submembranes. Gephyrin is critical for anchoring and clustering the glycine receptor in the postsynaptic densities by attaching to the β -subunits of the GlyRs and to the intracellular microtubules (see Box Fig. 12.1) [Fritschy, et al., 2008].

Such a role appears analogous to that of the ankyrin family of proteins, which restrict lateral mobility of many membrane proteins, including transporters and channels. Gephyrin or related proteins are also associated with $GABA_A$ receptors. Another protein in the scaffolding complex is collybistin, which interacts with gephyrin. Collybistin is thought to induce the submembrane clustering of gephyrin into aggregates that in turn recruit GlyRs to the postsynaptic site opposite the presynaptic release sites (Kins et al., 2000; Kneussel & Loebrich, 2007; Xiang et al., 2006). The specific localization of these receptor complexes opposite inhibitory transmitter release is actually initiated by the presynaptic adhesion molecule neuroligin2 (NL2), which specifically drives inhibitory postsynaptic membrane differentiation. NL1 serves the same function for excitatory postsynaptic specialization (Levinson, et al., 2010; Poulopoulos et al., 2009). NL2, produced in the presynaptic glycinergic or GABAergic neuron, binds the postsynaptic gephyrin (presumably penetrating the postsynaptic membrane) and functions as a specific activator of collybistin, thus guiding the membrane localization of the receptor. Deletion of NL2 in mice perturbs both glycinergic and GABAergic transmission and leads to loss of postsynaptic specializations (postsynaptic densities or PSD), specifically at perisomatic inhibitory synapses (Poulopoulos et al., 2009). Antisense treatment and gene knockout experiments indicate that gephyrin is essential for the clustering of glycine receptors and certain $GABA_A$ receptor subtypes at developing inhibitory synapses. In addition, in peripheral organs, gephyrin has an enzymatic function in the biosynthesis of the molybdenum cofactor, a coenzyme of oxidoreductases.



BOX FIG. 12.1 **Model of gephyrin-dependent glycine receptor (GlyR)**. Trafficking/anchoring. Gephyrin (light orange) links the GlyR in the membrane to the intracellular cytoskeleton (microtubules and microfilaments) for trafficking to the surface, clustering at synapses and dispersion of clusters during plasticity.

GLYCINERGIC NEUROTRANSMISSION AND NEUROLOGIC DISEASE (*cont'd*)

How does collybistin induce the submembrane gephyrin aggregations?

Collybistin II is a GDP/GTP exchange factor (GEF) co-localized with gephyrin (Xiang, et al., 2006). Collybistin is activated via NL2 and in turn activates the GTPase Cdc42 of the Rho/Rac family of small G proteins. It is thought that the activation of Cdc42 is involved in the submembrane scaffold/actin filament organization that is specific for the membrane tethering of the glycine and GABA receptors (Kins et al., 2000). Cdc42 is known to be involved in regulating cytoskeletal dynamics, reorganizing actin filaments, assembly–disassembly of intercellular junctions and conveyance of signals to intracellular processes relating to polarity, migration, vesicle trafficking and cytokinesis (Heasman & Ridley, 2008; Sinha & Yang, 2008). The specificity and localization are determined by the particular activated GEF, in this case collybistin, which is activated by NL2. The latter is extruded from the presynaptic transmitter release site to adhere to the precise postsynaptic receptor site.

Site-directed mutagenesis studies are informative concerning GlyR subunit domains involved in pentamer assembly and interaction with cytoskeleton

These experiments also contributed to understanding the agonist-binding pocket and ion channel domains. For example, α-subunits can produce functional homopentameric channels in recombinant expression systems, while β-subunits cannot. The α−homomeric glycine receptors are sensitive to the $GABA_A$ antagonist picrotoxin and may represent extrasynaptically located receptors. In contrast, heteromeric αβ-GlyRs are insensitive to picrotoxin and are clustered at synaptic sites. Current studies of the subunit stoichiometry are most consistent with a ratio of 2α:3β, but this still requires confirmation (Grudzinska et al., 2005). Mutagenesis of certain amino acid residues in the extracellular domain of β-subunits, making them similar to α -subunits, confers the ability to assemble into homomeric channels. Mutagenesis of other amino acid residues that differ between α and β subunits has identified domains involved in binding glycine and Zn^2, a metal ion that modulates glycine receptor function (Lynch, 2009). The molecular structures of the GlyR subunits, the molecular changes of Cys-loops in channel conductance, and the structures of gephyrin and associated proteins (neuroligin, neurexin) in the scaffolding for GlyRs and GABA receptors are described in detail by (Dresbach et al., 2008).

Distribution of Glycine Receptors

Immunocytochemical mapping specific for GlyR-α-subunits reveals their colocalization with [^{3}H]strychnine

Although most glycine receptors are found in a subset of retinal neurons, spinal cord and brainstem, a small but significant population is located in more rostral brain regions. Interestingly, β-subunit mRNA is abundant in many brain structures and even non-neural tissues, where neither [^{3}H] strychnine binding nor known α subunit mRNAs are found, suggesting that β-subunit glycine receptor proteins may have yet unknown functions (Lynch, 2009). While the bulk of data indicates that in adult animals heteromeric α1β−GlyRs are the main glycine receptors, excitatory nonsynaptic GlyRs in embryonic neurons contain α2. Immunocytochemical experiments suggest the presence of α2β, α3β and α4β at glycine synapses of adult mouse retina. Electrophysiological and immunocytochemical studies indicate that α3β-receptors mediate glycinergic inhibitory transmission in the nociceptive sensory neurons of spinal cord dorsal horn. The latter are implicated in signaling of inflammatory pain in the spinal cord (Harvey et al., 2004). Of considerable interest are recent physiological studies showing GlyR function in hippocampus. Strychnine-sensitive presynaptic GlyRs are found on hippocampal mossy fiber terminals early in postnatal development (Kubota et al., 2010) and α3-GlyRs are located preferentially associated with glutamatergic nerve endings in hippocampus (Eichler et al., 2009). Extrasynaptic GlyRs in the hippocampus are found to exert a tonic inhibitory role and data showing glycine modulation of NMDA receptor function may indicate important roles of glycine in regulating hippocampal network functions implicated in epilepsy, memory and learning (Xu & Gong, 2010).

Detailed anatomic studies of autopsied human brain and spinal cord have been revealing

In the forebrain, GlyRs are most abundant in the substantia nigra (about 80% of pigmented neurons), particular interneuronal subtypes in the striatum (those characterized by choline acetyltransferase, parvalbumin or calretinin), and are detectable scattered throughout the globus pallidus and medullary laminae regions around the lateral and mesial segments of the globus pallidus. In spinal cord, the most intense immunoreactive staining is observed in the dorsal and ventral horns, the spinal trigeminal tract and nucleus, inferior olivary complex, dorsal motor nucleus of the vagus and hypoglossal nucleus. In the substantia nigra and striatum, the presence or absence of GlyRs differentiates neuronal subgroups. GlyR immunoreactivity was found on plasma membranes of both soma and dendrites. Distributions in human brain were in agreement with those reported in rat brain. Gephyrin was found widely distributed in human brain and spinal cord and a large proportion, but not all, of GlyRs in brainstem and spinal cord show punctate immunoreactivity co-localized with gephyrin. Since high-resolution microscopy analysis suggests that not all GlyRα1 subunit co-localizes with gephyrin, it is thought that other mechanisms are involved in GlyR localization, presumably at presynaptic or extrasynaptic sites (Baer et al., 2009). Functional studies in rodents have demonstrated that glycine application can stimulate strychnine-sensitive acetylcholine (ACh) (Darstein et al., 2000) and dopamine (Yadid et al., 2010) release in striatum. These data together with the anatomic data in human brain implicate glycine in basal ganglia function.

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Glycine and GABA may be co-utilized as neurotransmitters at distinct subsets of inhibitory synapses

Immunoelectron microscopy revealed glycine receptor antigen apposed to nerve terminals that contain the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD), which constitutes a highly reliable marker for GABAergic neurons (see Ch. 18) (Triller et al., 1987). Furthermore, co-release of GABA and glycine at single postsynaptic sites has been demonstrated by electrophysiological recording in slice preparations from spinal cord and has been shown to result in the simultaneous activation of both $GABA_A$ and glycine receptors (Jonas, et al., 1998). As glycine and $GABA_A$ receptors differ in their elementary channel conductances and kinetic response properties, glycine and GABA co-release may allow the formation of postsynaptic response properties that are distinct from those of purely glycinergic or GABAergic synapses, respectively. The variations in the subunits for both receptors have evolved in a manner to permit intricate coordination of their functions. In the spinal cord, for example, inhibitory transmission by both glycine and GABA is thought to be crucial in the coordinated activation of muscles during locomotion. This coordination involves controlling temporal and spatial activation patterns for each muscle, agonists and antagonists, at each joint bilaterally (Nishimaru and Kakizaki, 2009).

In summary, there is considerable evidence to suggest that glycine signaling impacts GABAergic, as well as with glutamatergic, cholinergic and dopaminergic networks in multiple regions of CNS including hippocampus, basal ganglia, brainstem, retina and spinal cord, as described in the foregoing sections.

Molecular Genetics Elucidates Pathophysiology of Startle Disease

Human startle disease, hyperekplexia, is related to genes controlling glycinergic transmission

Investigations of genes whose protein products control glycinergic neurotransmission led to the elucidation of properties of the inhibitory glycinergic synaptosomal membrane organization, as well as the pathophysiology of hyperekplexia. This disease is a rare, but potentially fatal, inherited disorder characterized by markedly abnormal startle responses to sudden, unexpected stimuli, particularly auditory, but also somatic. The responses consist of exaggerated muscle stiffness, sometimes with forced eye closure and flexion of neck, trunk and limbs. There may be falling over "like a stiff stick" without loss of consciousness. The electroencephalogram does not exhibit electrical seizure activity during such episodes, so this is not considered an epileptic disease. From a physiologic viewpoint, the startle responses and stiffness are thought to be an exaggerated release from a state of reduced glycinergic inhibition in the brainstem and spinal cord (Andermann et al., 1980; Chung et al., 2010; Davies et al., 2010).

The first indications that this disease may be due to failure of glycinergic inhibition were derived from studies of mice in which genes related to glycinergic receptors were knocked out to study glycine transmission. It was noticed that the mice showed phenotypical responses similar to the human hyperekplexia (HPX) phenotype (see references in (Davies et al., 2010)). These early findings led eventually to discovery in affected people of mutations in five different genes: glycine receptor subunit-α1 (GlyRα1), glycine receptor subunit-β (GlyR β), presynaptic glycine transporter (GlyT2), and the genes for the postsynaptic glycine receptor accessory proteins gephyrin and collybistin.

Postsynaptic Gene Mutations

Mutations in GlyRα1 account for about 80% of HPX

Over 30 distinct GlyRα1 mutations among more than 100 index case families in several international surveys have been identified. About 70% of cases show recessive inheritance, a few percent exhibit compound heterozygote inheritance and the remainder show dominant inheritance (Harvey et al., 2008). In order to determine the effects on function of the receptor mutations, wild type (WT) or mutant human GlyRα1 genes were expressed in HEK293 cells. These studies indicated that recessive mutations produced decreased numbers of functional channels that could convey glycine-mediated currents, but biotinylation of cell surface proteins disclosed that the cell surface expression of receptors was reduced while the whole cell expression was comparable to that of the WT GlyRα1(Villmann et al., 2009). Thus, the defect in these mutant glycine receptor proteins is in their trafficking and/or integration into the postsynaptic membranes. The products of most of the recessive mutants are unstable; they are retained in the ER and appear to enter the proteasome pathway for degradation (Chung et al., 2010; Villmann et al., 2009). Mutations in the β-subunit have also been identified in HPX and in exaggerated startle (Rees et al., 2002).

In contrast to effects of the recessive mutations, the dominant inheritance mutations alter ligand-gated ion channel functions including glycine binding, ion channel gating and conductance, as well as desensitization of the receptors. Dominant forms of HPX have been linked to GlyRα1 mutations resulting in amino acid substitutions in transmembrane segment, which forms part of the inner wall of the ion channel pore, or adjacent regions (see Structure and Function of Glycine Receptors above).

Mutations in postsynaptic accessory proteins perturb glycinergic receptor localization

These mutations are in the genes for gephyrin (Rees et al., 2003) and collybistin (Jedlicka et al., 2009). These proteins are

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critical to trafficking receptors to the postsynaptic membrane and anchoring them opposite presynaptic transmitter release sites (see under Gephyrin section above). Studies of mutations that produce HPX helped elucidate these gene functions.

It is of considerable interest to the study of refractory epilepsy and other hippocampal functions that knock-out of collybistin (Jedlicka et al., 2009) or NL2 decreases inhibitory circuit activity and increases excitability of cortical neurons in mouse hippocampus along with reducing the numbers of gephyrin and $GABA_AR$ clusters on somata of granule cells (Jedlicka et al., 2010). One might expect that mutations that alter the function of NL2, collybistin or gephyrin would have an impact on the treatment of epilepsy or other functions if inhibitory circuits in the hippocampus are reduced or abolished. In fact, mutations have been discovered in the human gene for collybistin in a patient with symptoms of both hyperekplexia and epilepsy [35] and in a patient with epilepsy and emotional and cognitive disturbances (Kalscheuer et al., 2009). However, these have been seen only in single individuals.

Mutations in Presynaptic Glycine Transporter 2

Glycine is transported from synaptic clefts into astrocytic processes enwrapping the synaptosome or into the presynaptic axon terminal by Na,Cl-dependent symporters of the SLC6 superfamily (see Chap. 3). GlyT1 (SLC6A9), which is in astrocytic membranes, is mainly responsible for preventing glycine from diffusing out of the particular synapse into other synapses, controlling extracellular glycine, modulating glycine at excitatory glutamate receptors and terminating glycine inhibitory transmission (Eulenburg et al., 2010). GlyT2 (SLC6A5) is in presynaptic neuron membranes and is responsible for terminating glycine transmission at the inhibitory sites and for taking up the glycine back into the axon terminal to replenish the transmitter supply.

Defects in GlyT2 in HPX were suggested by the finding that knockout mice null for the GlyT2 transporter exhibit phenotypes similar to that of human HPX. R. J. Harvey, M.I. Rees and coworkers (Harvey et al., 2008), noting a "relative dearth of well-characterized postsynaptic targets" for all their subjects with HPX, focused on screening for mutations in all 16 coding exons of the SLC6A5 gene in an international cohort of 83 sporadic and familial HPX patients devoid of mutations in the postsynaptic genes described above. They reported a mosaic of missense and nonsense mutations in SLC6A5 in 23 of the cases, leaving about 60 single, unrelated, sporadic cases of HPX with no genetic diagnosis. In the majority of mutations, GlyT2 mutations were inherited as compound heterozygotes, indicating that that SLC6A5 is predominantly inherited as a recessive trait. One case of dominant inheritance was observed.

Subcellular localization studies were performed with WT and mutant GlyT2 genes transfected into Xenopus oocytes. These studies revealed that GlyT2 mutations result in defective subcellular localization of the transporter and/or decreased glycine uptake. Some mutations affected glycine and Na^+ binding to the transporter (see Harvey et al., 2008 for references). A number of other genes involved in trafficking, targeting, and recycling of GlyT2, and in concentration of glycine within vesicles or presynaptic release of glycine, remain to be investigated in view of the many HPX patients that currently have no identified genetic cause (Chung et al., 2010; Davies et al., 2010). These authors also point out the possibility that defects in the gene for or the activity of the astrocytic GlyT1 might be a cause of glycine encephalopathy in some patients in whom there is no deficiency in the glycine cleavage system (see nonketotic hyperglycinemia in Chap. 42).

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Evidence suggests that the activity of nNOS can be increased by brain lesions or ischemia (see Chap. 35). As a signaling molecule, NO differs from conventional neurotransmitters in that (i) it is not present in synaptic vesicles, (ii) it is not released by exocytosis and (iii) no specific extracellular synaptic receptors for NO exist. Rather, NO is released upon stimulation and diffuses from its site of production, which can be either neuronal or glial cells, to affect neurons up to 100 μm away. At these locations, NO activates a soluble form of guanylyl cyclase through electron transfer to the heme group on the enzyme. The increase in cGMP concentration subsequently activates cGMP-dependent protein kinases. In the CNS, NO has been speculated to play a role in both long-term potentiation (LTP) and long-term depression, although the precise mechanism remains to be defined. One counterargument for a role of NO in LTP is the observation that the latter persists in transgenic mice lacking nNOS. Evidence suggests that NO can directly S-nitrosylate a diverse array of intracellular proteins (Jaffrey et al., 2001; Sen & Snyder, 2010).

Other gaseous molecules (sometimes termed gasotransmitters) may also act as intercellular messengers. One putative candidate is carbon monoxide, which is generated from the conversion of heme to biliverdin, a reaction catalyzed by an oxygenase. One form of the enzyme, heme oxygenase-2 (HO-2), is constitutive and is found in high concentrations in various brain regions. HO-2 is activated by calcium-calmodulin, as are nNOS and eNOS. Like NO, CO increases the production of cGMP. Recently, attention has been focused on hydrogen sulfide (H_2S), a chemically reactive substance with toxic actions, as an additional putative gasotransmitter (Gadalla & Synder, 2010; Sen & Synder, 2010). H_2S, which is present in the brain at ~100μM concentrations is synthesized by the enzyme, cystathionine β-synthase. H_2S can modify enzymes post-translationally via sulfhydration, a process that results in their activation. Although there is clear evidence that H_2S acts as a vasodilator in the vascular system, its functional significance in the CNS has yet to be defined. However, roles for H_2S in long-term potentiation, induction of Ca^{2+} waves in glia and as a neuroprotectant have been suggested (Gadalla & Snyder, 2010).

Astrocytes also play a pivotal role in signaling events at the synapse

Traditionally, glial cells have been thought to play an inert structural role in the support of neuronal activity. However, it is now abundantly clear that, in addition to the pre- and postsynaptic elements of the synapse, astrocytes constitute a third signaling element in what has now been termed the 'tripartite synapse' (Perea & Araque, 2005). Astrocytes express a wide range of receptors for neurotransmitter, including those for glutamate, adenosine, norepinephrine, GABA, histamine and ACh. Many of these receptors are metabotropic and are linked to PLC activation, IP_3 production and the mobilization of intracellular Ca^{2+}. A consequence of the activation of astrocytic receptors that results from neurotransmitter release is that the astrocytes themselves release, in a Ca^{2+}-dependent manner, a number of 'gliotransmitters' such as glutamate, ATP, D-serine and tumor necrosis factor alpha (TNFα). These gliotransmitters can then provide feedback regulatory control of the neuron at both pre- and postsynaptic locations (Halassa & Haydon, 2010). For example, glutamate released from astrocytes is thought to regulate synaptic transmission at both excitatory and inhibitory synapses (Fellin, 2009). In addition, D-serine, a co-agonist at neuronal NMDA receptors, is synthesized exclusively within astrocytes and, following its release, activates synaptic NMDA receptors (Panatier et al., 2006). ATP is also released from astrocytes and can either interact with purinergic receptors or, alternatively, be degraded to adenosine, which can then elicit a tonic suppression at hippocampal synapses (Pascual et al., 2005). Astrocytes may also play a role in 'synaptic scaling', a phenomenon observed following prolonged periods of altered synaptic activity. For example, when the neuronal activity of cortical or hippocampal pyramidal cells is blocked by tetrodotoxin, the amplitude distribution of miniature excitatory postsynaptic currents is scaled upwards. The cytokine, TNFα, which is released from astrocytes, is postulated to increase the population of neuronal AMPA receptors at the cell surface, which in turn increases synaptic strength (Stellwagen et al., 2006). Collectively, these studies suggest that astrocytes play a central role in the modulation of synaptic signaling events following neurotransmitters release.

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