CHAPTER

7

Intracellular Trafficking

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OUTLINE

INTRODUCTION

The basic mechanisms of membrane trafficking and the functions of the biosynthetic secretory and endocytic pathways in the nervous system will be examined. First, the fundamental steps involved in vesicle formation will be defined. Second, the diverse biosynthetic secretory pathways and their functions will be examined, including biogenesis of organelles, delivery of plasma membrane components, and secretion. Third, endocytic pathways will be summarized including receptor-mediated endocytosis, membrane recycling and signaling. Finally, the specialized processes of synaptic vesicle trafficking serve to integrate these two pathways and represent a highly specialized form of membrane trafficking in the nervous system.

GENERAL MECHANISMS OF INTRACELLULAR MEMBRANE TRAFFICKING IN MAMMALIAN CELLS INCLUDE BOTH UNIVERSAL AND HIGHLY SPECIALIZED PROCESSES

Eukaryotic cells have evolved a complex intracellular membrane organization. This organization is partially achieved by compartmentalization of cellular processes within specialized membrane-bounded organelles. Each organelle has a unique protein and lipid composition. This internal membrane system allows cells to perform two essential functions: to *sort and deliver* fully processed membrane proteins, lipids and carbohydrates to specific intracellular compartments, the plasma membrane, and the cell exterior, and to *uptake* macromolecules from the cell exterior (reviewed in Derby & Gleeson, 2007; van Vliet et al., 2003). Both processes are highly developed in cells of the nervous system, playing critical roles in the function and even survival of neurons and glia. The *biosynthetic secretory pathway* is responsible for protein sorting and delivery and allows, among other functions, for cell–cell communication through secreted products. This delivery process starts at the endoplasmic reticulum (ER) and finishes in the cell plasma membrane or, in some cases, in specific intracellular organelles. To accomplish this, specific proteins must be properly directed to the correct destination, while other proteins are retained as residents within specific organelles along the way.

The series of molecular events responsible for the uptake process constitutes the *endocytic pathway*, which enables cells to internalize macromolecules from the cell exterior, forming an endosome. The endosome is an intermediate organelle that serves as an essential component for many receptor-mediated signaling pathways and as a sorting compartment for eventual delivery to a specialized organelle known as the lysosome. Once in the lysosomal lumen, digestive enzymes provide essential metabolites from these macromolecules (i.e., free amino acids and lipids) directly to the cytosol for its use.

Intracellular membrane compartments are in a continuous, balanced and dynamic interaction. A constant, directed flow of membrane components among different compartments occurs at all times in a single cell. Transport vesicles of variable shape and composition provide the means by which membrane components and soluble molecules, generically referred as cargoes, move from a donor to a target compartment (Fig. 7-1). Such vesicles first bud off from the donor membrane and later fuse with the target membrane. In order to maintain the biochemical composition and the unique structural identities of each organelle, eukaryotic cells developed specialized retrieval and retention mechanisms. The retrieval process implies that recycling transport vesicles must take only the proteins to be recycled (i.e., proteins that are continuous residents in their original compartment, or resident proteins), while others are left behind. The specific sequence of events observed in any direction within the biosynthetic and the endocytic pathways implies that transport vesicles need to fuse only with the correct target organelle. These mechanisms allow for maintenance and proper balance of membrane flow among compartments, assuring that each protein is targeted for the correct location as well as redirecting resident membrane components to the compartment where they originated.

Neurons constitute the most striking example of membrane polarization. A single neuron typically maintains thousands of discrete, functional microdomains, each with a distinctive protein complement, location and lifetime. Synaptic terminals are highly specialized for the vesicle cycling that underlies neurotransmitter release and neurotrophin uptake. The intracellular trafficking of a specialized type of vesicles in the presynaptic terminal, known as synaptic vesicles, underlies the ability of neurons to receive, process and transmit information. The axonal plasma membrane is specialized for transmission of the action potential, whereas the plasma membrane of the neuronal cell body and dendrites is specialized to receive signals from other nerve cells.

Glial cells also require extensive, albeit less diverse, intracellular membrane trafficking activity. This includes the

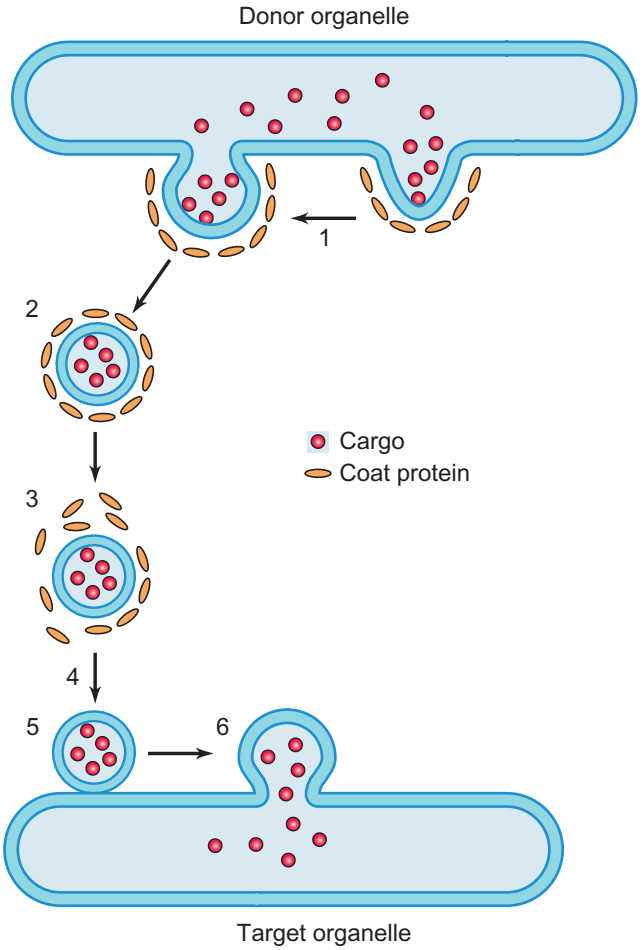

FIGURE 7-1 **Fundamental steps of intracellular membrane transport.** A series of basic steps *(1 to 6)* allows the transfer of material (generally referred to as cargo) from the lumen and membrane of a donor compartment to a target/acceptor compartment. First, specific cargo (red circles) is selected for packaging in the donor organelle (*1*). This process is concurrent with the formation of a specific protein coat (yellow ovals) on the cytosolic surface of the donor membrane, which helps mold a newly formed transport vesicle. Once a transport vesicle is formed, it buds off from the donor organelle (*2*). Transport vesicles shed their protein coats shortly after budding (*3*) in an active process that involves either an ATPase/chaperone or a small GTPase. After uncoating, vesicles are actively translocated across the cytoplasm, usually through mechanisms mediated by microtubule-based molecular motors (*4*). Transport vesicles eventually reach, recognize and tether to the appropriate target organelle *(5)*. Finally, unloading of the transport vesicle cargo to the target membrane occurs by membrane fusion (*6*).

delivery of myelin-associated proteins during both the generation and maintenance of myelin. In the case of oligodendrocytes, different processes that myelinate different axons may require differential trafficking of these components, similar to the situation in neurons (see Ch. 4). Finally, astrocytes must distribute a variety of transporters and channels to different astrocytic domains as part of their role in regulating the neuronal environment and delivering nutrients. Intracellular trafficking of membrane components thus plays a central role in both neuronal and glial cells.

FUNDAMENTALS OF MEMBRANE TRAFFICKING ARE BASED ON A SET OF COMMON PRINCIPLES

Although the various membrane compartments utilize a different repertoire of proteins for coat formation, vesicle budding and fusion, the basic principles behind these processes are similar (Fig. 7-1). A number of ordered, regulated molecular events allow for protein segregation into discrete, separate membrane domains (Derby & Gleeson, 2007; van Vliet, et al., 2003). Although these events are essential for functionality and survival of all cell types, they are highly specialized and often more complex in neuronal cells. The process of vesicle budding is initiated by the selection and assembly of specific components in donor membranes, which need to be packaged and delivered together to the same target membranes. The formation of specific protein coats on the cytosolic face of the donor membrane gathers these common components into a discrete membrane domain that can be packaged as a transport vesicle. This sequence of events provides for membrane targeting specificity.

Most transport vesicles bud off as coated vesicles, with a unique set of proteins decorating their cytosolic surface

The vesicle coat has two major known functions. First, it concentrates and selects specific membrane proteins in a discrete portion of donor organelle membrane that will serve as a seed for the formation of transport vesicle. Second, the assembly of coat proteins into curved structures delineates the area of the forming transport vesicle. The size and curvature is a function of the coat composition. Thus, vesicles with similar vesicle coat have closely similar size and shape (Kreis et al., 1995).

Ultrastructural and biochemical studies first suggested a higher degree of diversity within these vesicle types (Kreis et al., 1995). In addition to the coated vesicle types familiar from electron microscopy, video microscopic imaging in living cells identified tubulovesicular carriers, rather than spherical-shaped vesicles, budding off from endosomes, the ER and the *trans*-Golgi network. Transport vesicles important for longer-distance trafficking, such as secretory vesicles, synaptic vesicle precursors and other specialized vesicle types, are discussed in detail later in this chapter. There are at least three well-established types of coated vesicles common to all cell types, each having a characteristic coat protein composition, and each used for different transport steps in the cell.

Clathrin-coated vesicles mediate transport from the Golgi apparatus to endosomes, and from the plasma membrane to endosomes (Schmid, 1997). A multi-subunit protein, clathrin, constitutes the major protein of this vesicle type (see Ch. 2). Clathrin is composed of three large and three small polypeptide chains, which assemble to form a triskelion (Fig. 7-2). Regulatory mechanisms control the assembly and formation

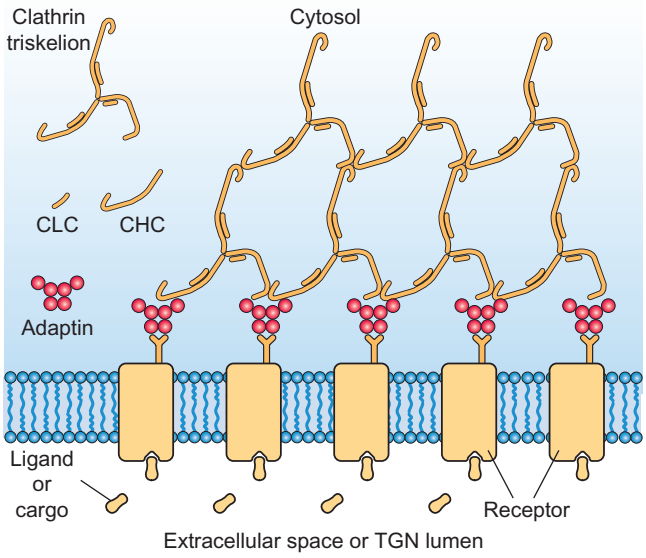

FIGURE 7-2 Initial formation of clathrin-coated vesicles. Clathrin coats are the best characterized and understood pathway for the formation of vesicles either from the plasma membrane or from the *trans* Golgi network (TGN) lumen (Rappoport et al., 2004). Clathrin consists of three heavy chains (CHC) that join near their C-termini to form a triskelion. Three light chains (CLC), of undetermined function, associate with the proximal segments of the heavy chains. Ligands (on the extracellular space) or protein cargo (in the TGN lumen) bind to their specific transmembrane receptor, inducing a conformational change permitting its cytoplasmic domain to interact with adaptins (i.e.; AP1 for the plasma membrane, AP2 for the TGN). Clathrin binding to the adaptins then induces clathrin triskelion assembly and eventually the coated pits that develop into clathrin-coated vesicles. Auxillin (not shown) is another component of the triskelion that appears to be important for eventual removal of clathrin coats by a member of the Hsp70 chaperone family.

of a convex, polyhexapentagonal basket-like structure by these triskelions. This structure is responsible for the formation of coated pits on the cytosolic face of plasma membranes. Another major protein component of clathrin-coated vesicles, generally known as adaptins, bind and link clathrin coats to the membrane. Multiple types of adaptins have been described, each type binding a unique set of cargo receptors and associated to a specific membrane organelle. Different sets of adaptins participate in forming the coat assembly complexes for the Golgi (AP1) and the plasma membrane (AP2) (Fig. 7-2). Sequential assembly and interactions between adaptins and clathrin aid in bud formation. Adaptins also bind and recruit transmembrane proteins known as cargo receptors, which in turn bind and recruit soluble proteins in the membrane lumen. This provides a means by which selected proteins may be packaged into a clathrin-coated vesicle.

Coat-protein purification first lead to the identification of a complex composed of seven individual coat-protein subunits, known as COPI or coatomer. COPI-coated vesicles mediate intra-Golgi transport and Golgi-to-ER retrograde transport (Hsu & Yang, 2009). The coats of these vesicles do not show the geometric forms seen with clathrin coats and have a more complex protein composition. Some of these subunits bear a sequence similarity to clathrin adaptors. In addition, there is a small GTP-binding protein, ADP-ribosylation factor 1 (Arf1), present on COPI-coated vesicles.

COPII-coated vesicles are anterograde transport vesicles used early in the secretory pathway, budding from the endoplasmic reticulum (Barlowe, 2002; Gurkan et al., 2006). COPII components were initially identified by using yeast mutants showing secretion defects and subsequently isolated from mammalian cells. The appearance of COPII coats in electron micrographs is readily distinguished from that of both clathrin and COPI coats. COPII-coated vesicles bud from the ER for transport to the Golgi, and their coat is composed of four individual coat-protein subunits. One of these is a small GTP-binding protein, SarI, which exhibits some sequence similarity to Arf1.

GTP-binding proteins, such as small monomeric GTPases and heterotrimeric GTPases (G proteins) facilitate membrane transport

GTPase proteins regulate a variety of cellular processes, including membrane trafficking in cells (Stenmark, 2009). These proteins act as switches by alternating between an active, GTP-bound state and an inactive, GDP-bound state. Various regulatory proteins modulate the rate of conversion between these two states. Guanine-nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, resulting in GTPase activation. On the other hand, GTPase-activating proteins (GAPs) promote GTP to GDP hydrolysis, thus inactivating GTPases.

Although both heterotrimeric G proteins and monomeric GTPases appear to have roles in the regulation of vesicle transport, the role of monomeric GTPases is better understood (Stenmark, 2009). In particular, coat protein assembly is regulated by coat-recruitment GTPases, which are monomeric GTPases. In general, coat-recruitment GTPases are abundant in the cytosol, where they exist in an inactive, GDP-bound state. Hydrolysis of GTP by small GTPases typically results in their translocation to membranes. Examples of such coat-recruitments GTPases are ADP-ribosylation factor (ARF) proteins, which regulate clathrin and COPI coat assembly at Golgi membranes, and the SarI protein, which regulates COPII coat assembly at the ER. COPI vesicle budding for example, is triggered by the hydrolysis of GTP to GDP by membrane-associated ARF1. Once in the membranes, ARF1–GTP recruits preassembled coatomers, resulting in membrane deformation. The cytoplasmic tail of an abundant transmembrane protein, known as p24, binds to COPI through an internal KKXX sequence, promoting vesicle formation (Lee et al., 2004). As explained below, KKXX sequences represent a motif that is instrumental in retrieval mechanisms leading to retrograde transport of proteins in the secretory pathway.

Another set of proteins regulates the budding of COPII-coated vesicles through modulation of small GTPase activity. Before COPII-coated vesicles bud from the ER, a specific membrane-embedded GEF binds to SarI and catalyzes the exchange of GDP to GDP by SarI. A conformational change is induced in SarI after this GTP–GDP exchange, leading SarI to expose a fatty acid tail normally hidden in the SarI core.

Exposure of the fatty acid tail allows SarI to insert at the ER membrane and recruit COPII subunits, which in turn start the membrane deformation and the vesicle budding process (Barlowe, 2002; Gurkan, et al., 2006). GTP hydrolysis by SarI and other GTPases can eventually reverse this process, albeit at a slow rate in the absence of a GAP activity. This suggested that coat-recruitment GTPases might regulate temporal aspects of vesicle assembly working by hydrolyzing GTP at a slow but predictable rate.

Dynamins are involved in pinching off of many vesicles and membrane-bounded organelles

The dynamin superfamily of proteins is associated with diverse cellular activities including vesicle scission, membrane-bounded organelle division and fusion, cytokinesis and cellular antiviral protection mechanisms (Praefcke & McMahon, 2004). Dynamins represent a heterogeneous group of large GTPases, distinct from the small, Ras-like GTPases. Dynamins and dynamin-related polypeptides feature a conserved, larger GTPase domain of ~300 amino acids, a middle domain and a GTPase effector domain (GED). These three domains are critical for the arrangement of oligomeric complexes (dimers or tetramers) that promote their GTPase activity. The oligomerization-dependent activation, the capacity to interact directly with membrane lipids, and their low GTP binding activity makes dynamins unique and different from the small Ras-like GTPases (Praefcke & McMahon, 2004). In addition to the three characteristic domains described above, individual members of the dynamin superfamily display distinctive targeting motifs including plecktrin homology (PH) domains responsible for their association to negatively charged lipid membranes, proline-rich domains (PRDs) that bind to SRC-homology-3 (SH3) domains, and/or specific mitochondria-targeting domains.

In mammals, classical dynamins include dynamins 1, 2 and 3. Dynamin 1 is enriched within the brain and localizes to presynaptic terminals. Dynamin 2 has a ubiquitous tissue distribution, whereas dynamin 3 is localized in testis and the brain, where is found postsynaptically (Praefcke & McMahon, 2004). Classical dynamins are known to be involved in vesicle scission. As budding proceeds, clathrin-coated vesicles recruit, among other proteins, dynamin through their PRD and PH domains. Dynamin and other binding partners form a complex that bends the membrane at the neck of the budding vesicle. This is achieved by locally distorting the membrane or by changing the lipid composition through the actions of lipid-modifying enzymes, which are recruited to the dynamin complex. Once in close proximity, the two membranes leaflets fuse, resulting in the final pinching off of the coated vesicle, an activity known as 'pinchase' activity. The classical dynamins are not only responsible for the pinching off of clathrin-coated vesicles, but are also involved in budding of non–clathrin-coated vesicles such as caveolae (Henley et al., 1998) and in phagocytosis (Gold et al., 1999). The relevance of dynamins to neuronal function is highlighted by mutations in dynamin 2, which result in dominant intermediate Charcot-Marie-Tooth disease, a neurodegenerative disorder affecting motor and sensory neurons (Durieux et al., 2010) (See Box 7-1).

MUTANT DYNAMIN 2 AND NEUROMUSCULAR DISORDERS

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The DNM2 gene located on human chromosome 19 encodes for dynamin 2, a ubiquitously expressed protein. As observed for other classical dynamins, the dynamin 2 protein features an amino terminal GTPase domain responsible for GTP hydrolysis, a middle domain (MD) involved in its self-assembly; a pleckstrin homology domain (PH), which binds with specific phospholipids; a GTPase effector domain (GED) that participates in self-assembly and GTPase activity regulation; and a C-terminal proline-rich domain (PRD) that binds to SH3 domain-containing binding partners (Praefcke & McMahon, 2004). Functionally, dynamin 2 has been shown to associate with nascent clathrin-coated pits in a helical fashion, and induce "pinching off" or release of vesicles in a GTP-depending dependent manner. Accordingly, it appears to be involved in clathrin-independent endocytosis during the formation of phagosomes and caveolae as well as in micro- and macropinocytosis. Dynamin 2 also localizes to the Golgi apparatus, where it may participate in the vesicular sorting machinery by regulating the trafficking of vesicles from the trans-Golgi network (TGN). The binding of DNM2 to actin-binding proteins including Abp1, and cortactin, which is an important element of the clathrin-mediated endocytosis machinery, highlights the relevant role that DNM2 plays in regulating actin-based endocytosis, vesicle formation, and trafficking.

Based on the known cellular functions of dynamin 2 is involved on, it is not surprising that mutations in this protein could result in disease (Durieux et al., 2010). Indeed, it has recently been genetically determined that mutations in DNM2 lead to centronuclear myopathy (CNM), a congenital myopathy, as well as two forms of Charcot-Marie-Tooth (CMT), an autosomal dominant peripheral neuropathy. To date, many DNM2 mutations have been reported that result in CNM (Susman et al., 2010). Histopathologically, CNM is characterized by centrally located nuclei in muscle fibers. CNM manifests as a progressively deteriorating disorder featuring delayed motor milestones; facial and generalized muscle weakness, which can results in inability to run in childhood; ptosis (or "drooping eyelid"); and ophthalmoplegia. Some patients also develop pes cavus (or raised arch) even before symptomatic muscle weakness presents (Susman et al., 2010). CNM patients with DNM2 mutations showed a mild axonal neuropathy without reduction in motor conduction velocities, while others will show reduced nerve conduction velocities (NCV). Clinical severity ranges from typical mild forms to very

MUTANT DYNAMIN 2 AND NEUROMUSCULAR DISORDERS (*cont'd*)

severe forms showing onset at infancy. The molecular basis for such variability in severity of symptoms is unknown.

Charcot-Marie-Tooth (CMT) disease is a set of relatively common progressive genetic peripheral neurological disorders with five classes linked to more than 40 genes (Patzko et al., 2011). CMT is characterized by muscular weakness and atrophy, pes cavus foot deformity, depressed tendon reflexes, sensory loss and prominent axonopathy (Niemann et al., 2006). In some reported cases, axonal neuropathy is also associated with asymptomatic neutropenia (abnormally low number of neutrophils) and early-onset cataracts. Several point mutations in DNM2 that result in CMT have been reported (Durieux et al., 2010). Intriguingly, some mutations result in Charcot-Marie-Tooth type 2 (CMT2), whereas others lead to a variation of CMT known as dominant intermediate Charcot-Marie-Tooth (DI-CMT). Electrophysiologic studies on some dynamin-2-associated CMT patients show intermediate or axonal motor median NCV at or near normal values (≥45 m/s), while other mutations that cause DI-CMT show NCVs ranging from normal to almost 50% reduced. The majority of mutations in DNM2 leading to CMT are located in the PH domain, with while two other mutations are mapped on the MD and the PRD.

The ubiquitous tissue expression of dynamin 2 and the multiple cellular functions proposed for this protein raised many important questions on the pathogenic mechanisms underlying CMT and CNN. Why are specific cell types affected? How do different mutations (sometimes located immediately adjacent to each other) in the same gene result in different diseases? A molecular basis underlying the increased vulnerability of selected cell types to DMN2 mutations is currently unknown, but likely results from alterations in the functional specializations of these neuronal cell types. In this regard, it has been proposed that DNM2 mutations may affect protein trafficking and axonal transport of vital material for the axons (Patzko et al., 2011) (see Ch. 8). It is unclear why deficits in axonal transport induced by different mutant versions of dynamin 2 would differentially affect muscle cells and sensory/motor neurons, but differences in the composition, amounts, and regulation of axonal transport in these cell types have all been documented.

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The dynamin-like superfamily of proteins includes, among others, dynamin-like proteins (Dlps), which lack the PRD and are instead involved in mitochondrial fission–fusion events (Labrousse et al., 1999). Optic atrophy 1 (OPA1), an evolutionary conserved dynamin-like protein expressed from yeast to humans, contains an amino-terminal mitochondrial import sequence and localizes between the inner and outer mitochondrial membranes, where it is thought to help mitochondrial fusion. Mutations in this OPA1 result in dominant optic atrophy (DOA), an inherited human neuropathy of the retinal ganglion cells (Ban et al., 2010). Mitofusin is another evolutionarily conserved Dlp, which localizes to the cytoplasmic side of the outer mitochondrial membrane. Mitofusin has been proposed to regulate fusion mitochondrial fusion and dynamics (Santel, 2006). Curiously, mutations in mitofusin 2 result in axonal neuropathy with optic atrophy (Zuchner et al., 2006).

Removal of coat proteins is catalyzed by specific protein chaperones

Once vesicles are released from the donor membrane, chaperones of the Hsp70 family remove the clathrin coat (Schmid, 1997). These chaperones are ATPases recruited and activated in a regulated manner by specific protein domains present in the membrane-associated protein auxillin. In neuronal cells, clathrin coats need to be removed before transport vesicles undergo active translocation to the axonal processes from the Golgi apparatus. Presumably, uncoating allows membrane proteins exposed on the surface of vesicles to be recognized by molecular motors of the kinesin superfamilies (see Ch. 8). COPI and II coats also need to be removed to permit active translocation by molecular motors and to allow fusion of transport vesicles with a target membrane. GTP hydrolysis by the appropriate coat-recruitment GTPase causes COPI and COPII coats to disassemble. This step is catalyzed by the specific GAP protein ARF-GAP in the case of COPI vesicles, and sec23 protein in the case of COPII vesicles (Kreis et al., 1995).

The cytoskeleton appears to have a significant role in the localization of different organelles of the biosynthetic pathway, as well as in the transport of vesicles between different organelles (see Chs. 6 and 8). Both microtubules (MTs) and actin filaments appear to be involved in these processes. For example, through its association with the minus end of MTs, the Golgi apparatus is localized in close proximity with the centrosome.

MTs also provide the tracks used by molecular motors for transport of vesicles and organelles throughout the neuron (see Ch. 8). Neuronal cells are highly dependent on these MT-based transport systems because of their high degree of polarization and their complex architectural dynamics. The actin cytoskeleton also appears to play a role in vesicle tethering and transport, particularly in the event of regulated secretion, and in the synaptic vesicle cycle. Finally, cytoplasmic-linker proteins, or CLIPs, appear to mediate certain types of interactions among vesicles and MTs. CLIP-170 for example, is proposed to link endosomal membranes to MTs.

SNARE proteins and rabs control recognition of specific target membranes

Rabs are a family of monomeric GTPases, normally localized on the cytosolic face of membranes, which aid in vesicular transport specificity (Pfeffer & Aivazian, 2004). There are more than 30 known Rabs in mammals, each showing a distinctive organelle distribution. Targeting to specific organelles is thought to be mediated by variable carboxyl terminal sequences, which appear to bind proteins specific to the surface of each organelle (Zerial & McBride, 2001). In their active, GTP-bound state, Rabs appear to bind other proteins in the membrane (generally known as Rab effectors) through an exposed lipid anchor.

Soluble N-ethylmaleimide sulfhydryl factor attachment protein receptors (SNAREs) provide specificity to a target membrane and participate in the fusion process. SNAREs are transmembrane proteins with a cytoplasmic helical domain. There are more than 20 different SNAREs in each cell, each one associated with a specific membranous organelle (Pelham, 2001). SNAREs exist as complementary sets of v-SNAREs (in the transport vesicles) and t-SNAREs (in the target membranes). The SNARE hypothesis proposes that SNARE molecules are the basis for specificity of membrane fusion. When the appropriate complement of v- and t-SNAREs bind, their cytoplasmic helical domains interact to form coiled-coil interactions, which are thermodynamically highly stable. These interactions result in the formation of a stable, four-helix bundle complex known as a *trans*-SNARE pair that brings the two membranes together (Brocker et al., 2010). There is a high level of specificity in the interaction between v- and t-SNAREs. Three SNAREs in one membrane and one in the other appear to be needed to form a functional complex containing four-helix bundle. If different SNARE combinations display different specificities, this explains how a single SNARE can be involved in several transport steps. Inhibitory proteins provide another level of regulation by binding to t-SNARES, limiting their amount and availability for fusion. SNARE complexes are best characterized in neuronal cells, where they mediate the docking and fusion of synaptic vesicles to the presynaptic plasma membrane, both critical steps necessary for the process of neurotransmitter release (see below). Specific SNAREs in the nerve terminal can be targeted and cleaved by various botulinum neurotoxins, highly specific proteases that block synaptic transmission (Montal, 2010).

The *trans*-SNARE pair becomes the *cis*-SNARE pair after fusion (this name is given because after fusion, they are in the same membrane) and disassembly of the *cis*-SNARE complex is necessary for the SNAREs to be reused. A soluble ATPase named N-ethylmaleimide-sensitive fusion protein (NSF) catalyzes this step. NSF acts as a chaperone, actively unfolding the coiled-coil interaction between SNAREs with the help of adaptor proteins (Brocker, et al., 2010). Once *cis*-SNAREs are disassembled, v-SNAREs may be returned to the donor membrane for reuse. The regulated disassembly of SNARE complexes by NSF provides the means to control the degree and amount of membrane fusion within cells.

Rabs are also thought to regulate v-/t-SNARE recognition, and ultimately vesicle fusion. Rabs and their effectors may modulate vesicle trafficking through different means. Although each Rab member has a discrete localization, a common feature of all Rabs is that they contribute to vesicle tethering near the target site and to the release of SNARE inhibitory proteins, thus facilitating v- and t-SNARE recognition. Once GTP is hydrolyzed, the inactive Rab protein moves to the cytosol and becomes available for another transport cycle (Pfeffer & Aivazian, 2004). GTP exchange factor (GEF) and GTPase activating protein (GAP) proteins regulate the otherwise intrinsically low GTPase activity of Rabs (Barr & Lambright, 2010). In addition, GDP-dissociation inhibitor (GDI) and GDI-displacement factor (GDF) may regulate the association of Rabs with membranes. These proteins are effectors of pathways activated by specific extracellular signaling events, thus linking specific transport steps to extra-cellular stimuli. Finally, some Rabs appear to directly link specific organelles and transport vesicles to the cytoskeleton.

Unloading of the transport vesicle cargo to the target membrane occurs by membrane fusion

The membrane fusion event does not necessarily happen immediately after vesicle docking. Depending on the target, fusion may be constitutive or regulated. Fusion events are often subject to exquisitely sensitive regulatory mechanisms; i.e., localized Ca^{2+} influx at the presynaptic terminal (see below). Although docking indicates sufficient membrane proximity for proteins to interact, membrane fusion requires a much higher degree of proximity. Membranes must be brought as close as 1–2 nm for lipids to move from one membrane to another. Specific proteins facilitate this high-energy-demanding process by removing water molecules from the cytosolic face of closely opposite membranes. SNARE complex formation appears to help repel water as coiled-coil structures wrap around each other. Although *in vitro* experiments indicate that SNAREs could allow for slow membrane fusion by themselves, accessory proteins appear to assist and regulate membrane fusion events *in vivo* (Bonifacino & Glick, 2004).

Fusion events may be homotypic or heterotypic. Homotypic fusion indicates fusion between membranes that originate from the same compartment (i.e., fusion of ER-derived vesicles to form tubular vesicular clusters, see below). Heterotypic membrane fusion indicates fusion of membranes originating from different compartments (i.e., synaptic vesicles and the plasma membrane). Triggered fusion, such as in neurotransmitter release, is typically heterotypic (Bonifacino & Glick, 2004).

THE BIOSYNTHETIC SECRETORY PATHWAY INCLUDES SYNTHETIC, PROCESSING, TARGETING AND SECRETORY STEPS

The biosynthetic secretory pathway starts at the rough endoplasmic reticulum with synthesis of components, continues through the Golgi apparatus, and eventually finishes in the cell plasma membrane, secretory vesicles, or lysosomes. Along the way, a variety of processing and packaging events must occur. To facilitate understanding of each step, a functional description of each organelle along the biosynthetic pathway is useful before discussing specific transport steps within this pathway.

Historically, endoplasmic reticulum has been classified as rough or smooth, based on the presence (RER) or absence (SER) of membrane-associated polysomes

The biosynthetic pathway of membrane trafficking begins with the rough endoplasmic reticulum (RER). Membrane protein synthesis occurs on the cytosolic surface of the ER (Fig. 7-3). Shortly after initiation of synthesis, proteins destined for secretion, for service as integral membrane proteins or for residence along the biosynthetic pathway are targeted to the ER lumen by a specific amino-terminal sequence. As new polypeptide chains start to protrude from the ribosomes, they are recognized by the signal recognition particle (SRP), which targets the newly formed polypeptide chain to the cytosolic side of the ER membrane by means of a specific SRP receptor and a protein complex known as Sec61p (Rapoport, 2007). Soluble luminal or secretory proteins are cotranslationally translocated into the ER lumen, through the Sec61p membrane channel, while transmembrane proteins are cotranslationally inserted into the ER membrane directly.

Once in the ER lumen, proteins oligomerize and fold. Proper folding in most cases is facilitated by various isomerases and chaperones resident in the ER. Complex mechanisms exist within the ER to ensure that proteins are correctly folded and multimeric protein complexes correctly assembled before leaving this organelle. For example, some cargo proteins destined to move through the biosynthetic secretory pathway are actively recruited and concentrated at specific membrane domains where vesicles form. The recruitment of these proteins is mediated by exit signal sequences located on their properly folded surface. ER chaperone proteins, such as BiP, recognize and cover such exit signals in incomplete or improperly folded proteins, thereby impeding their exit from the ER (Dudek et al., 2009). Such defective proteins may be sent to the cytosol, where they are degraded through the action of the proteasome. In some cases, such as the acetylcholine receptor, 90% or more of the synthesized protein is degraded before it reaches the plasma membrane. Thus, chaperone-mediated quality control mechanisms prevent defective proteins from reaching their normal destinations where they might interfere with the function of normal proteins (Trombetta & Parodi, 2003). A number of human diseases are related to defects in this quality control mechanism, including cystic fibrosis, Tay-Sachs disease (see Ch. 19), and Charcot-Marie-Tooth disease type 1a (see Ch. 38) (Aridor & Hannan, 2000; Ni & Lee, 2007).

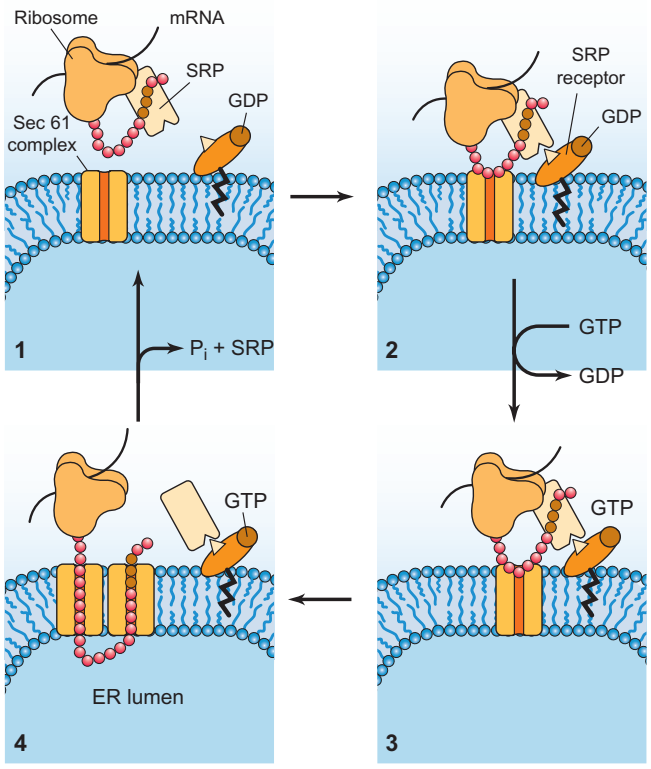

FIGURE 7-3 Insertion of proteins into the ER membrane. Initiation of membrane protein insertion into the endoplasmic reticulum. *(1)* Signal-recognition particles (SRP) associate with peptide signal sequences (brown residues) of nascent membrane proteins (red residues). *(2)* These complexes associate with SRP receptors in the endoplasmic reticulum membrane, which contain bound GDP. *(3)* Bound GDP is exchanged for cytoplasmic GTP, and *(4)* translocation of peptides through the Sec61 protein complex (or protein translocator) occurs as GTP is hydrolyzed. The peptides are oriented N- to C-terminal–outward as they insert through a membrane.

The covalent addition of sugars (or glycosylation) is an important part of the proper folding of many membrane-associated proteins, and this activity represents one of the major functions of the SER (Table 7-1) (Shental-Bechor & Levy, 2009). The majority of proteins destined for the ER itself (both membrane and soluble ER proteins), secreted proteins, and proteins destined to other organelles in the biosynthetic secretory pathway are in fact sugar-modified proteins, or glycoproteins. Addition of different sugar complexes may be important for the normal function of a protein or for targeting a protein to a particular organelle, but the first stages of glycosylation follow a typical pattern.

Initially, a 14-sugar oligosaccharide containing *N*-acetylglucosamine, mannose and glucose, known as the precursor oligosaccharide, is added *en bloc* to the side chain

TABLE 7-1 Compartmentalization of Glycosylation Processing Steps in the Secretory Pathway

Compartment	Processing step	Enzymes
Endoplasmic reticulum	• XN-linked glycosylation • Removal of mannose • Removal of 2 glucoses	• ER mannosidase • Glucosidase I • Glucosidase II
cis-Golgi network	• Phosphorylation of oligosaccharides in lysosomal luminal proteins • O-linked glycosylation	• N-acetylglactosaminyl transferase
cis-Golgi cisterna	• Removal of mannose	• Golgi mannosidase I
medial Golgi cisterna	• Removal of mannose • Addition of N-acetylglucosamine	• N-acetyl glucosamine transferase I
trans-Golgi cisterna *trans*-Golgi network	• Addition of galactose • Addition of NANA • Sulfation of tyrosines and carbohydrates • Addition of sialic acid • Selective recognition of M6P by M6P receptor	• Golgi mannosidase II • α2,6-sialyltransferase

Immunoelectron microscopic and biochemical fractionation data on specific processing enzymes have permitted the localization of specific processing steps in the processing pathway for proteins in the Golgi complex. The distribution of many remaining enzymes and steps had not yet been determined. The distribution for each step indicated in this table reflects a significant enrichment, rather than an exclusive localization.

amino group of an asparagine residue of nascent proteins. Only asparagines within the consensus sequence NXS/T are modified (where X is any amino acid except proline). This process is also known as N-linked glycosylation, and it is catalyzed by the action of a membrane-bound enzyme known as oligosaccharyl transferase. Proteins in the cytosol are not subject to this kind of modification, because the oligosaccharyl transferase enzyme is oriented in the ER membrane with its catalytically active site facing the ER lumen. Since most proteins are cotranslationally imported into the ER, N-linked oligosaccharides are almost always added during protein synthesis. It is thought that N-linked glycosylation is used as a mechanism for recognizing the extent of protein folding, allowing only properly folded proteins to leave the ER.

Most glycoproteins have one mannose and three glucoses removed by specific enzymes while still in the ER. This sugar trimming process has a role in the correct folding of N-linked glycoproteins (Parodi, 2000). For example, specific chaperones such as calnexin and calreticulin bind to monoglucosylated (trimmed) oligosaccharides and assist in their folding. Enzymatic hydrolysis of the remaining glucose in the monoglucosylated protein species leads to dissociation of these chaperones. Proteins not properly folded are enzymatically reglucosylated, recognized again by calnexin or calreticulin and retained in the ER. This process allows the protein to follow the right folding sequence. Improperly folded proteins that cannot be recovered are eventually translocated back to the cytosol, where they are deglycosylated, ubiquitinated and degraded in proteasomes. The abnormal accumulation of unfolded proteins in the ER can eventually trigger a pathway known as 'unfolded protein response,' which leads to the transcription and translation of specific genes that help the ER to continue its appropriate function (Rutkowski & Kaufman, 2004).

The ER plays a major role in the synthesis of lipids needed for the generation and maintenance of membranes used by organelles in the secretory and endocytic pathways (including the Golgi apparatus, secretory vesicles, lysosomes, endosomes, and the ER itself), as well as lipid components in mitochondrial, peroxisomal and plasma membranes. Certain cell types specialized in lipid synthesis (i.e., steroid hormone-producing cells and hepatocytes) feature an extensive smooth endoplasmic reticulum (SER) network, which is characterized by the lack of associated ribosomes. Chapter 5 describes in details the biosynthesis of lipids by the ER. Finally, Ca^{2+}-binding proteins within the ER help control intracellular Ca^{2+} levels. This important aspect of the ER function is discussed in detail in Chapter 24.

Biosynthetic and secretory cargo leaving the ER is packaged in COPII-coated vesicles for delivery to the Golgi complex

COPII-coated vesicles exit the ER from specialized regions of the ER membrane devoid of bound ribosomes, known as 'ER exit sites' (Fig. 7-4). These domains are specialized in the generation of COPII-coated transport vesicles (Gurkan et al., 2006). Soon after budding from the ER, COPII-coated vesicles lose their coat and fuse homotypically to form structures known as vesicular tubular clusters, transitional ER, or ER–Golgi intermediate compartments (ERGICs). ERGIC represents an independent ER-derived compartment, which lacks ER-resident proteins (Appenzeller-Herzog & Hauri, 2006)

Two models have been proposed to explain the process of packaging and transport of COPII vesicles from the ER towards the Golgi: the bulk flow transport and selective transport models. Bulk transport proposes that only signals necessary for retention and recycling play a role in the selectivity of cargoes. Proteins without such signals follow a default pathway, which send them to the Golgi apparatus.

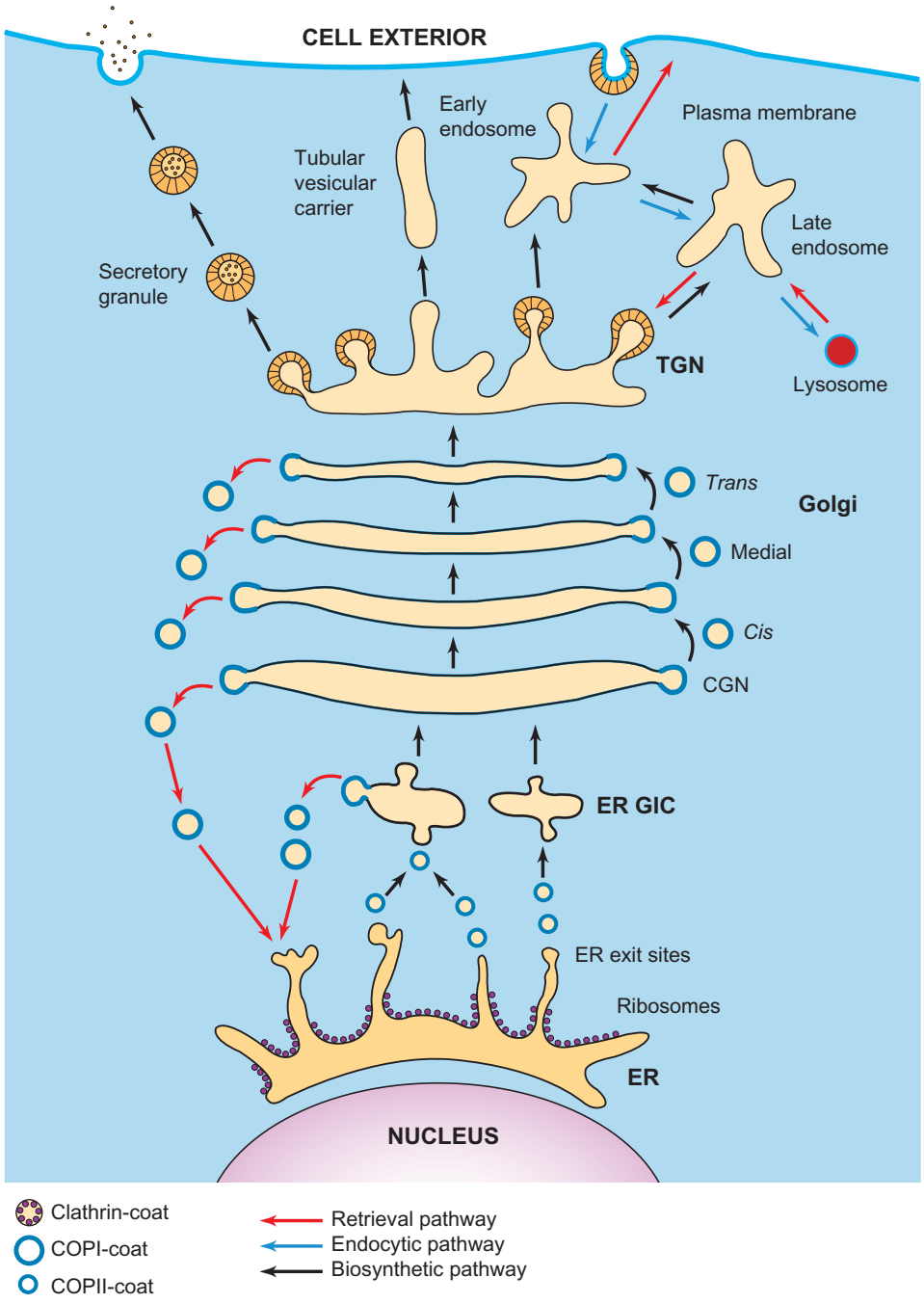

FIGURE 7-4 **Schematic illustrating intracellular compartments and major transport steps along the secretory and the endocytic pathways.** Intracellular membrane-bounded organelles transfer material to each other in the form of transport vesicles. Different coat proteins help in the selection of specific cargoes during different transport steps. In the secretory pathway (black arrows), cargo proteins start their journey by budding off from ER exit sites, ribosome-free areas of the endoplasmic reticulum (ER). These proteins are packaged in COPII-coated vesicles, which later fuse to form vesicular clusters (ERGIC). Several ERGICs merge and fuse to form the *cis*-Golgi network (CGN). COPI-coated vesicles mediate the recycling of ER proteins and are also thought to mediate the transport of cargoes between Golgi stacks (see Figure 7-5). Once in the *trans*-Golgi network (TGN), proteins get sorted to the plasma membrane, or in some cases to lysosomes. Resident proteins of each organelle along the secretory pathway achieve their localization through specific retrieval mechanisms (*red arrows*). Different types of coated vesicles and tubulovesicular carriers transport cargo from the TGN to their final destinations. For example, some cargoes reach the plasma membrane and/or the cell exterior by means of secretory granules, while others appear to travel in tubulovesicular structures or trough recycling endosomes. In the endocytic pathway (*blue arrows*), molecules are internalized in the form of plasma membrane-derived, clathrin-coated vesicles. These vesicles are delivered to the early endosomes, then to late endosomes, and eventually to lysosomes, where their content is typically degraded. Retrieval pathways (*red arrows*) allow for the recycling of proteins from the early endosome to the plasma membrane surface, and from the late endosome to the Golgi. Some differentiated cell types have additional pathways in addition to the general ones depicted in this figure, including specialized, extremely rapid secretory and recycling pathways for synaptic vesicle proteins in presynaptic terminals.

The selective transport model proposes that proteins are concentrated before packaging by a process involving the recognition of specific exit signals. This model is consistent with the fact that approximately 90% of ERGICs are recycled back to the ER, suggesting active concentration of soluble and membrane proteins from recycling vesicles. With some few exceptions, exit signals directing proteins outside ER to Golgi are not well understood, although some cargo proteins are actively recruited to vesicles through exit signals on their surface. These exit signals are recognized by receptor proteins, which also interact with components of the COPII coat. The possibility exists that abundant proteins could leave the ER via bulk transport, while certain low-abundance proteins would be packaged by selective transport.

Specific retrieval and retention mechanisms allow for the sustained maintenance of the ER's biochemical composition. Shortly after being formed, vesicular tubular clusters start budding COPI-coated vesicles, which retrogradely carry ER-resident proteins and proteins involved in ER budding reaction back to the ER. Thus, the ERGICs sort out proteins by either recycling ER proteins or sending secretory cargo to the *cis*-Golgi. This recycling process, known as retrieval transport, is essential for the maintenance of biochemical identity of specific membrane compartments. As a result of retrieval transport, vesicular tubular clusters change their composition as selected proteins are returned to the ER (Teasdale & Jackson, 1996). The retrieval of ER resident proteins depends in turn upon retrieval signals, some of which bind directly to COPI coats, thereby getting packaged in COPI-coated vesicles. Examples of retrieval signals are the KKXX and H/KDEL amino acid sequences (both names based on the single-letter amino acid code) (Maattanen et al., 2010).

The KKXX sequence is present at the carboxyl terminus of ER membrane proteins, while the H/KDEL is present in soluble ER proteins, such as the aforementioned chaperone BiP (Dudek et al., 2009). Soluble ER proteins containing these sequences bind to receptor proteins such as the Golgi-localized H/KDEL receptor. The KDEL receptor is a transmembrane protein that recognizes and recruits H/KDEL-containing proteins into COPI-coated vesicles that undergo retrograde transport. H/KDEL receptors need to cycle themselves between the ER and the Golgi. To achieve this, H/KDEL receptors' affinity towards H/KDEL containing proteins needs to change from very low binding affinity while in the ER to a very high binding affinity while in vesicular tubular clusters and Golgi. Such differences in binding affinities are achieved due to pH-dependent conformational changes in the H/KDEL receptor. Whereas the pH in ER is near neutral, H^+ pumps in the Golgi create slightly acidic conditions and result in high-affinity binding of H/KDEL-containing soluble proteins by H/KDEL receptors.

Another potential mechanism for protein retention is ER proteins binding to each other to form large complexes that cannot be recruited in transport vesicles (Teasdale & Jackson, 1996). This mechanism is known as 'kin recognition.' Because the concentration of resident ER proteins can be very high (some have been estimated in the millimolar range), it can be achieved even with low-affinity interactions among resident proteins.

The Golgi apparatus is a highly polarized organelle consisting of a series of flattened cisternae, usually located near the nucleus and the centrosome

Camillo Golgi first discovered the Golgi apparatus while studying neuronal cells, using a staining technique based on the use of heavy metals (Farquhar & Palade, 1998). Neurons have the most abundant Golgi complement as a result of the high rate of synthesis and processing of membrane proteins in neurons. Golgi localization has been shown to depend on intact microtubules and the activity of microtubule-based molecular motors. Each Golgi stack or cisterna has an entry (or *cis*) face, and an exit (or *trans*) face. The Golgi apparatus usually consists of three to eight cisternae, and it can be divided morphologically and functionally into *cis*-, medial and *trans*-Golgi (Glick & Nakano, 2009). This heterogeneity was demonstrated by the differential distribution of processing enzymes within individual cisternae, particularly for enzymes involved in the glycosylation process (Table 7-1).

The *cis* and *trans* faces of the Golgi apparatus are each associated with a specific compartment composed of interconnected tubular and vesicular structures respectively known as the *cis*-Golgi network (CGN) and the *trans*-Golgi network (TGN). The CGN is thought to originate through the fusion of multiple vesicular clusters. Sorting of proteins is an important process that takes place in these two compartments. After entering the CGN, proteins can either be retrieved to the ER or continue their travel through the Golgi apparatus (Jackson, 2009). Similarly, within the TGN proteins are sorted in different transport packages and sent to their final destinations (De Matteis & Luini, 2008). Such destinations include specific domains of the plasma membrane (i.e., dendritic or axonal domains), lysosomes, secretory vesicles or earlier compartments in secretory pathway. The TGN is functionally and morphologically distinct from the stack cisternae in a number of ways. For example, the coat composition of buds derived from TGN (clathrin) differs from those in the Golgi stacks (COPI). The TGN is also a location where specific protein modifications occur, including tyrosine sulfation, proteolytic processing and sialylation (Glick & Nakano, 2009).

Processing of proteins in the Golgi complex includes sorting and glycosylation of membrane proteins and secretory proteins

Many glycosidases and glycosyltransferases reside within the Golgi apparatus (Nilsson et al., 2009). The great diversity of N-linked oligosaccharide structures on mature glycoproteins results from both the trimming of the original precursor oligosaccharide originally added to proteins in the ER and the addition of further sugars (Fig. 7-3). The result is the formation of complex oligosaccharides and high-mannose oligosaccharides. These two differ in that high-mannose oligosaccharides have no new sugars added to them. High-mannose oligosaccharides contain two *N*-acetylglucosamines and many mannose residues, usually the original number added in the ER. Complex oligosaccharides can contain multiple *N*-acetylglucosamines and a variable number of galactose

and sialic acid residues. The formation of both complex and high-mannose oligosaccharides depends largely on their accessibility by processing enzymes in the Golgi.

In the Golgi, another mode of protein glycosylation is O-linked glycosylation. In this process, sugars are added to the OH groups of selected serine or threonine side chains by glycosyl transferase enzymes. Typically, *N*-acetyl galactosamine is added first, followed by a variable number of additional sugar residues, sometimes up to 10. Finally, the Golgi apparatus is an organelle with an important role in lipid biosynthesis (van Meer & Hoetzl, 2010). The enzyme sphingomyelin synthase catalyzes the production of both sphingomyelin and diacylglycerol from the products phosphatidylcholine and ceramide, previously generated in the ER (see Ch. 5). In addition, more-complex glycosphingophospholipids are generated in the Golgi. As these sphingolipids associate with ER-derived cholesterol, they are segregated from unsaturated glycerolipids in the lipid bilayer. This results in a *cis*- to *trans*-Golgi sphingolipid/cholesterol gradient, which in turn translates into increased bilayer thickness. This membrane thickening could serve for the selective retention of some Golgi enzymes at the TGN. Golgi-derived vesicles are richer in cholesterol, which causes their lipid bilayers to be thicker than that of the Golgi membrane itself. Thus, only proteins with transmembrane regions long enough to span this thickness can enter transport vesicles at the TGN. This lipid-sorting model appears consistent with the fact that transmembrane domains of resident Golgi proteins are approximately five to six amino acids shorter than plasma membrane proteins (Derby & Gleeson, 2007; Munro, 2005).

The function of protein glycosylation is unknown for most proteins. However, some functional consequences had been established for the glycosylation of certain proteins (Scheiffele & Fullekrug, 2000). In some cases, glycosylation appears to help in the folding process in the ER or to serve a role in protein sorting. For example, carbohydrate binding proteins known as lectins help in ER-to-Golgi transport, as well as in the sorting process occurring in the TGN (Zanetta, 1998). In some cases, specific glycosylation patterns may serve to target a protein to a particular destination, such as the lysosome (see below). Alternatively, glycosylation can influence the stability and or turnover of a protein. Because sugar chains are not very flexible, they tend to protrude from the surface or glycoproteins, potentially limiting the approach of other molecules. For example, the high level of glycosylation on lysosomal membrane proteins makes them resistant to the action of lysosomal hydrolases.

Glycosylation also plays an important role in cell–cell adhesion (see Ch. 9). Such is the case of glycosylated cell-surface proteins that are recognized by specific lectins known as selectins. The high degree of glycosylation on proteoglycan core proteins also is important on the cell surface. Finally, many proteoglycans are secreted to become components of the extracellular matrix, and others remain attached to the plasma membrane (Ruoslahti, 1988, 1996).

Proteins and lipids move through Golgi cisternae from the *cis* to the *trans* direction

The addition of sugars to proteins occurs in an organized sequence in the Golgi (Schachter, 2000) (Fig. 7-4). Each cisterna is characterized by the abundance of specific processing enzymes. As proteins travel through different cisternae, they are gradually modified. In addition, each oligosaccharide-processing enzyme can only accept a glycoprotein as a substrate once a preceding enzyme has processed this protein. Therefore, the processing of proteins throughout the Golgi is ordered both spatially and biochemically. Enzymes catalyzing early steps are concentrated in the *cis* face of the Golgi, with the ones catalyzing downstream processing steps being localized closer to the *trans* face. This localization of specific processing steps in the Golgi apparatus and other organelles in the biosynthetic pathway is shown in Table 7-1. Finally, the lumen of the *trans* compartment appears to be continuous with the TGN, a station where sorting of proteins occurs to target plasma membrane, lysosomes or secretory vesicles.

How the Golgi apparatus maintains its polarized structure while molecules move from one compartment to another is still a matter of debate. Two models were originally proposed based on different kinds of experimental evidence (Fig. 7-5): the cisternal maturation model and the vesicular transport model. A third model known as the dual transport model combines elements from both vesicular transport and cisternal maturation models and can better explain intra-Golgi transport (Derby & Gleeson, 2007).

Morphological observations from electron microscopic images provided the basis for the cisternal maturation/progression model. In this model, vesicular tubular clusters from the ER reach the *cis* side of the Golgi apparatus as new cisternae. New *cis* cisternae continuously form and migrate through the Golgi stacks from *cis* to *trans* as they mature, in a manner analogous to a conveyor belt. Although everything moves forward, including enzymes (found mainly in early Golgi stacks), a specific distribution of processing enzymes within the Golgi is achieved by a continuous COPI-dependent retrograde vesicle flow. Newly formed cisternae receive their complement of proteins from the one immediately ahead, and later pass them backward to still newer cisternae at the next *cis* position. Documentation of *de novo cis*-Golgi formation, as well as the movement of large molecular structures such as procollagen, which would not fit in a transport vesicle, strongly supported this model (Bonfanti et al., 1998). However, a major difficulty faced by the cisternal progression/maturation model is explaining the origin of the TGN, an apparent steady-state compartment that contains a unique set of resident proteins and behaves differently from Golgi stacks in many respects.

The vesicular transport model was derived from observations that the Golgi is associated with many small vesicles and remains even after protein synthesis is switched off in the cell (Farquhar & Palade, 1998), suggesting that Golgi cisternae are stable. In the vesicular transport model, individual cisternae represent static structures, and cargo is transported through the Golgi in the form of transport vesicles, which bud from one cisterna and fuse with the next. The vesicular transport model more easily explains the differential distribution of processing enzymes within each Golgi compartment. Forward-moving proteins are selectively packed into forward-moving vesicles, while retrieved proteins are selectively packed and returned in retrograde-moving ones. Alternatively, vesicles move randomly and the flow occurs because of the continuous, specific

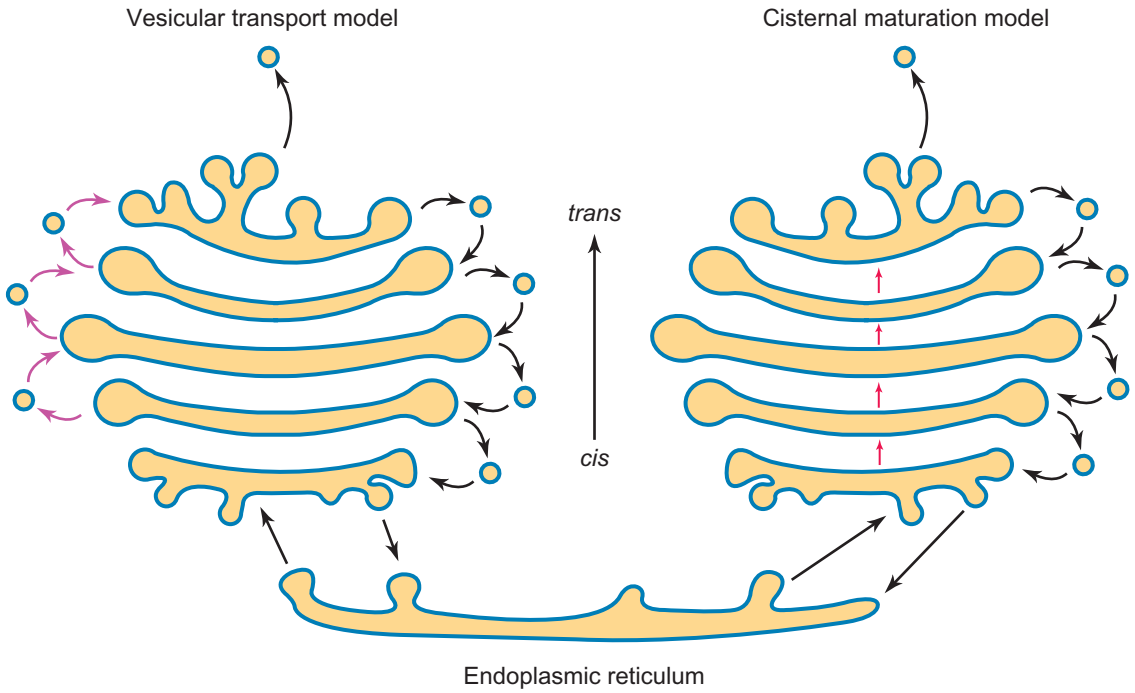

FIGURE 7-5 **Two models proposed for intra-Golgi transport.** In the vesicular transport model (left), cisternae are static, and each cisterna has a unique resident protein composition. Transport vesicles moving forward in the pathway (red arrows) provide the basis for the movement of molecules among cisternae throughout the Golgi. Retrieved proteins are selectively packed and returned in retrograde-moving vesicles. In the cisternal maturation model (right), individual cisternae mature as they move forward (red arrows) from the *cis* to the *trans* position in the Golgi. COPI-coated transport vesicles move resident proteins to the preceding cisterna, providing the means for specific localization of processing enzymes within the Golgi apparatus. Notice the absence of forward-moving transport vesicles in the cisternal maturation model. An alternative, dual transport model (not depicted here), combines elements of both models and is likely to represent intra-Golgi transport more accurately (see text).

input at the *cis* cisterna and the corresponding output at the *trans* cisterna. Experimental evidence suggested these cargo-carrying vesicles are COPI-coated vesicles. Movement of vesicles from one cisterna to another is helped by filamentous proteins, which restrict their movement and facilitate their fusion.

One problem with this model is that insufficient fusion markers have been identified to label each compartment. Such markers would be necessary for directional transport among different cisternae in the Golgi. In addition, COPI-coated vesicles appear to be the only type of transport vesicle observed within the Golgi, and they move only retrograde to the secretory pathway (Hsu & Yang, 2009). This evidence is difficult to reconcile with the anterograde movement of vesicles predicted by the vesicular transport model.

The dual-transport model combines aspects of the previous two models (Pelham & Rothman, 2000). Molecules so large that they would not fit in transport vesicles would move inside maturing cisternae. Bidirectional transport vesicles, on the other hand, would move proteins through the Golgi more rapidly. Molecular tethers would limit the movement of vesicles, promoting the transfer of cargo between adjacent cisternae, without the requirement for multiple sets of specific SNARE complexes. Regardless of the model proposed, proteins all complete their journey through the Golgi at the TGN, where they are sorted and packaged into specific transport vesicles that target them to specific cell domains and organelles (De Matteis & Luini, 2008).

Plasma membrane proteins are sorted to their final destinations at the *trans*-Golgi network

Soluble and plasma membrane proteins are delivered to axonal, dendritic or axo-dendritic plasma membrane domains by yet-unidentified mechanisms. Axonal and dendritic domains appear to be analogous to the apical and basolateral plasma membrane domain of epithelial cells (Higgins et al., 1997). The selective enrichment of membrane proteins at each of these neuronal domains can be achieved by means of selective protein transport delivery, selective endocytosis and specific retention. Certain proteins appear to be sent directly to each plasma membrane domain by a mechanism involving selective packaging of cargoes at the TGN and attachment to specific molecular motors. Apical and basolateral proteins have been observed to leave the TGN in different membrane carriers, and sorting signals have been identified within the primary sequence of some proteins that can direct them to apical or basolateral surfaces of epithelial cells. *In vitro* experiments first suggested that constitutive secretion was carried out entirely in the form of small vesicles. However, more recent imaging experiments using fluorescently tagged

proteins in living cells revealed a much more heterogeneous repertoire of membranes moving from the TGN towards the plasma membrane, including large tubulovesicular structures (Kaether et al., 2000; Lippincott-Schwartz et al., 2000).

However, not all proteins proceed directly to their eventual destination. Some proteins relocate from one plasma membrane compartment to another by means of transcytosis. Transcytosis involves endocytosis of selected proteins in one membrane compartment, followed by subsequent transport through early endosomes, recycling of endosomes and finally translocation to a different membrane compartment (i.e., from the apical to the basolateral surface). Sorting at the TGN and endosome recycling steps appear to have a primary role in the steady state distribution of proteins in different plasma membrane domains (Sampo et al., 2003). Additionally, selective retention of proteins at the plasma membrane by scaffolding proteins or selective removal may also contribute to normal distributions. Finally, regulatory mechanisms for microtubule-based molecular motors have been discovered that might explain the specific delivery of membrane proteins to discrete plasma membrane domains (see Ch. 8).

Lysosomal proteins are also sorted and targeted in the *trans*-Golgi network

Lysosomes are morphologically heterogeneous organelles whose major function is the degradation and digestion of macromolecules (see Ch. 43). They are found in all eukaryotic cells. The lysosomal membrane has a unique composition. ATP-dependent H^+ pumps maintain the low pH of the lysosomal lumen, and transporter proteins move sugars, amino acids and nucleotides resulting from digestion of macromolecules from the lysosomal lumen to the cytosol, where they can be either excreted or reused. The luminal side of this membrane compartment is filled with acid hydrolases, enzymes whose optimal pH is acid to 5.0. The near-neutral pH in cytosol provides a poor environment for the activity of acid hydrolases, thus protecting cytosolic components from hydrolases that might leak from lysosomes. Nearly 40 different types of lysosomal hydrolases have been described. These include glycosidases, lipases, nucleases, phospholipases and phosphatases. Genetic defects affecting the function of lysosomal hydrolases can lead to human disease, as they usually result in the accumulation of undigested material in lysosomes, often with severe pathological consequences (Saftig & Klumperman, 2009). Examples of these include Hurler's and Niemann-Pick diseases (see Ch. 43).

Several intracellular trafficking pathways converge at lysosomes

Hydrolases and lysosomal membrane proteins are delivered to lysosomes from the TGN (via late endosomes), whereas substances destined for digestion are provided to lysosomes by three different pathways: endocytosis, autophagy and phagocytosis (see below). Lysosomal membrane proteins and hydrolases are synthesized first in the rough ER and later transported to the TGN. In the TGN, they are packed in transport vesicles, which are delivered to late endosomes first, and subsequently to lysosomes. Specific targeting mechanisms ensure that these vesicles transport lysosomal proteins while excluding others. In the case of lysosomal hydrolases, this mechanism is based on the selective recognition of a mannose 6-phosphate (M6P) tag, a unique marker for proteins destined for lysosomes (Braulke & Bonifacino, 2009). M6P is added specifically to the N-linked oligosaccharides of soluble lysosomal enzymes as they pass through the lumen of the TGN. The signal to add M6P specifically to a glycoprotein is the presence of a motif in its primary sequence known as a 'signal dispatch.' The sequential action of two enzymes later catalyzes the addition of M6P to lysosomal hydrolases. The first is GlcNAc phosphotransferase, which binds the hydrolase and adds GlcNAc-phosphate to one or two mannose residues on each oligosaccharide chain. The second enzyme removes the GlcNAc residue, leaving a M6P behind. Most hydrolases contain multiple oligosaccharides, thus finishing with several M6P. This appears to facilitate the high-affinity signal of lysosomal hydrolases for the M6P receptor. A M6P receptor in the TGN recognizes and binds to M6P on the luminal side and to adaptins on the cytosolic side of assembling clathrin-coated pits in the Golgi (Ghosh et al., 2003). This allows selective packaging of lysosomal-directed proteins in the TGN for transport to a late endosome in the form of clathrin-coated vesicles.

The retrieval mechanism for the M6P receptor resembles the one previously described for the H/KDEL receptor (Braulke & Bonifacino, 2009). Optimal binding of M6P receptor to M6P occurs at pH 6.5–6.7, the pH found in the TGN. When transport vesicles arrive at late endosomes, the pH is lowered by the action of H^+ pumps. The affinity of the M6P receptor for its ligands is reduced at acid pHs, resulting in M6P receptor releasing the M6P in the late endosome. As a result, transport of lysosomal hydrolases occurs unidirectionally. Once the M6P receptor releases M6P-bearing hydrolases, the receptor can be returned to the TGN for reuse. Transport of the M6P receptor to either TGN or late endosome relies on signal peptides on the cytoplasmic tail region of the M6P receptor.

Under some circumstances, lysosomal hydrolases may fail to be properly packaged in the TGN, so they enter the default pathway to the cell surface, where they are secreted. Although these hydrolases do little harm at the nearly neutral pH of most extracellular fluids, they can also be returned to lysosomes by a pathway known as receptor-mediated endocytosis. In this pathway, M6P receptors are sent to the plasma membrane, where they bind escaped lysosomal hydrolases and bring them back to lysosomes through the early and late endosomes. Receptor-mediated endocytosis is a major component of the endocytic pathways for trafficking of membrane proteins and merits more detailed consideration.

Both constitutive and regulated neuroendocrine secretion pathways exist in cells of the nervous system

Vesicular proteins and lipids that are destined for the plasma membrane leave the TGN sorting station continuously. Incorporation into the plasma membrane is typically targeted

to a particular membrane domain (dendrites, the axon, pre- and postsynaptic membranes, etc.), but may or may not be triggered by extracellular stimuli. The eukaryotic cellular process called exocytosis is defined as the fusion of the vesicular membrane with the plasma membrane, leading to continuity between the intravesicular space and the extracellular space. Exocytosis carries out two main functions: it provides membrane proteins and lipids from the vesicle membrane to the plasma membrane and it releases the soluble contents of the vesicle lumen (proteins, peptides, etc.) to the extracellular milieu. Historically, exocytosis has been subdivided into constitutive and regulated secretion (Fig. 7-6); release of classical neurotransmitters at the synaptic terminal is a special case of regulated secretion.

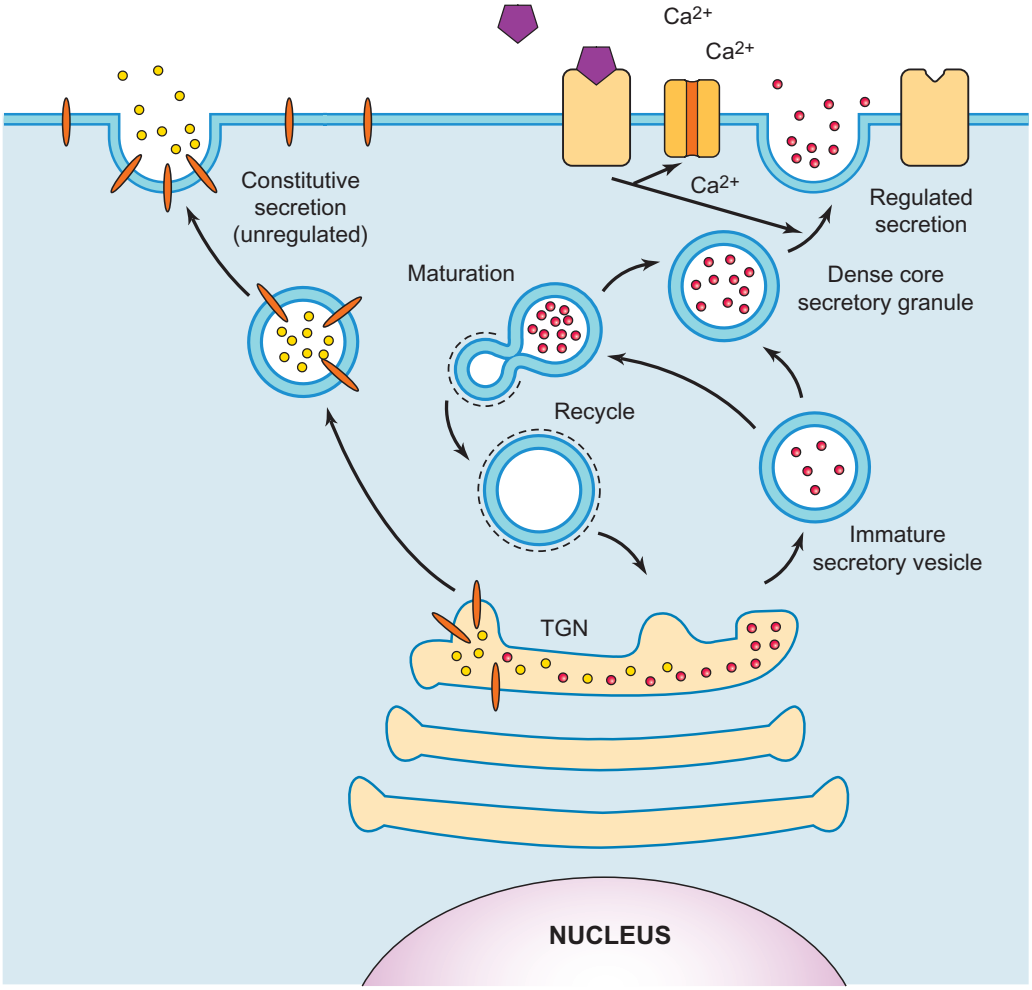

FIGURE 7-6 The constitutive and regulated secretory pathway. Eukaryotic cells determine the fate of newly synthesized proteins and lipids destined for the plasma membrane at the *trans*-Golgi network (TGN). Eukaryotic cells developed two different exocytic pathways, one called constitutive secretion, which is an unregulated or default pathway, and a second one, triggered by extracellular signals, which is known as a regulated secretory pathway because it is tightly coupled to extracellular stimuli. Lipids, secreted proteins and integral membrane proteins lacking a particular sorting signal are packaged into a common type of vesicles in the TGN. Once the transport vesicle buds off, it is steadily delivered to a common domain within the plasma membrane via the constitutive secretory pathway. This constitutive pathway typically provides the cellular plasma membrane with newly synthesized lipids and integral membrane proteins (*in blue*). In addition, some secreted proteins like extracellular matrix materials may be released constitutively as they are synthesized (*yellow circles*). Specialized secretory cells such as neurons and endocrine cells developed more selective exocytic pathways, known collectively as the regulated secretory pathway. These pathways form the basis for interneuronal communication. In this pathway, the exocytic event is triggered by a specific extracellular signal, allowing for a tight regulation of secretion. The regulated secretory pathway is mainly responsible for the release of specific cellular products such as hormones, peptides and neurotransmitters (in *red*). These cargoes are also sorted and concentrated in the TGN and packaged into immature secretory vesicles with a distinctive polypeptide composition. After the vesicle buds off of the TGN, they undergo a maturation process that is needed to concentrate their contents further and create a secretion-competent secretory granule. Secretory granules that contain protein or peptides often exhibit a dense core in electron micrographs and are thus called dense core secretory granules or vesicles. The maturation process often includes a series of steps that require formation of clathrin coats and recycling of membrane and membrane components to the TGN. Release of secretory granule contents requires binding of a ligand (*dark orange pentagon*) to a membrane receptor (*light orange*), which triggers movement of secretory granules closer to the plasma membrane and an influx of Ca^{2+} through a specific voltage-gated channel (*dark orange*) that is required for membrane fusion and release. A specialized variant of these pathways, which is extremely rapid, is found in presynaptic terminals.

The constitutive secretory pathway is also known as the default pathway because it occurs in the absence of a triggering signal

All eukaryotic cells possess an unspecialized exocytic pathway known as the constitutive secretory pathway. Vesicle membranes in this pathway fuse with the plasma membrane without any extracellular signal. Proteins secreted through this pathway are sorted into packages in which the constituents have a common destination. For example, molecular components of the active zone in the presynaptic membrane appear to be constitutively transported as part of a prepackaged dense core vesicle (Owald & Sigrist, 2009; Ziv & Garner, 2004). Thus, active zone scaffold proteins such as piccolo, bassoon and rim are present in the same transport vesicle, but synaptic vesicle proteins such as synaptophysin and synaptobrevin are not (Szodorai et al., 2009). Components of other functional microdomains in the neuronal plasma membrane such as nodes of Ranvier, fusion components, and the postsynaptic densities may be similarly packaged into discrete vesicles with unique destinations. This is likely to be a general feature of cells with specialized functions and distinct domains, including neurons and glia in the nervous system. However, the full complement of proteins needed in a particular membrane domain may not be necessarily packaged together in the TGN and local assembly of functional complexes, such as multisubunit ion channels, may still be necessary. The constitutive pathway has not been studied as intensively as regulated secretion. In particular, relatively little is known about targeting and regulatory mechanisms for these transport vesicles. Clathrin seems not to be directly involved in the constitutive secretory pathway, as antibodies that disrupt clathrin assembly *in vitro* inhibit endocytosis, but constitutive exocytosis is not affected.

Secretory cells, including neurons, possess a specialized regulated secretory pathway

Protein secretion is a multistep process that involves vesicle biogenesis, cargo loading, concentration and processing, vesicle transport and targeting, vesicle docking and Ca^{2+}-dependent vesicular fusion with the plasma membrane. In response to physiological demands, cells synthesize, concentrate and store secretory products into secretory vesicles, secretory granules or dense core vesicles (named because of their electron opaque content in electron micrographs). Exocytosis through regulated secretion helps accomplishing different cellular functions, including the release of hormones, peptides and neurotransmitters. Exocytosis through the regulated secretory pathway is also the primary basis for intercellular communication in the nervous system.

At least two classes of regulated secretion can be defined (Burgess & Kelly, 1987). The standard regulated secretion pathway is common to all secretory cells (i.e., adrenal chromaffin cells, pancreatic beta cells, etc.) and works on a time scale of minutes or even longer in terms of both secretory response to a stimulus and reuptake of membranes after secretion. The second, much faster, neuron-specific form of regulated secretion involves the release of neurotransmitters at the synapse. Release of neurotransmitters may occur within fractions of a second after a stimulus and neurotransmitter reuptake is on the order of seconds. Indeed, synaptic vesicles may be recycled and ready for another round of neurotransmitter release within 1–2 minutes (Morris & Schmid, 1995). These two classes of regulated secretion will be discussed separately after a consideration of secretory vesicle biogenesis.

Regulated secretion is easily recognized and distinguished from constitutive secretion. First, regulated secretion is linked to an extracellular stimulus, which in turn results in a transient local rise in intracellular Ca^{2+} that leads to fusion of the vesicle membrane with the plasma membrane and concomitant release of vesicle contents to the extracellular space. Second, the secretory products are concentrated into specialized vesicles that are morphologically distinct and abundant in secretory cells. Finally, the secretory vesicles are transported to cellular locations near release sites and significant numbers are subsequently stored in the cytoplasm as a ready releasable pool for further utilization. This means that secretory cells synthesize, package and store specific secretory products that are not released in significant amounts until a specific physiological stimulation is received.

Secretory vesicle biogenesis requires completion of a characteristic sequence of steps before vesicles are competent for secretion

Secretory vesicles in the regulated secretory pathway carry soluble proteins, peptides or neurotransmitters and are actively transported to selected subcellular domains for extracellular delivery in response to a specific extracellular signal. In secretory cells, the bulk of newly synthesized membrane and membrane-associated proteins may be devoted to generation of the secretory vesicles, sometimes referred to as secretory or dense core granules. Neurons in particular devote a large fraction of their biosynthetic activity to the synthesis and assembly of secretory vesicles. The extensive Nissl substance (mostly RER) and large Golgi complexes seen in neurons reflect this biosynthetic activity, and both structures were first described in neurons.

Neurons have multiple types of secretory vesicles whose secretion and biogenesis differ (Peters et al., 1991). The typical dense core vesicles (75–95 nm) deliver protein and neuropeptides to the extracellular space by the classic regulated secretory pathway; while more numerous smaller vesicles (40–50 nm) known as synaptic vesicles, specialize in the storage and delivery of small neurotransmitters such as acetylcholine, glutamate, glycine, gamma amino butyric acid (GABA) and monoamines. Most small, classical synaptic vesicles are electron lucent, but those containing noradrenalin may also have a dense core in electron micrographs. In contrast, secretory granules in endocrine or neuroendocrine cells may be significantly larger, as much as 165 nm in diameter.

The specific molecular signals that drive the packaging and aggregation of secreted proteins or their characteristic integral membrane proteins into distinct secretory vesicles within the TGN are currently unknown. Standard secretory vesicles emerge as immature vesicles from the TGN of the Golgi complex. The size of these immature secretory vesicles

is heterogeneous but all have a dense core. This core of aggregated proteins is separated from the vesicle membrane by a space filled with undetermined material, forming a halo-like structure. As secretory vesicles mature, they lose volume and surface area and their contents become concentrated as the result of coordinated retrieval of membrane that is recycled back to the late endosomes and TGN, and the internal vesicle acidification that occurs as a consequence of progressive concentration of protons by pumps in the vesicular membrane.

Biogenesis of synaptic vesicles in neurons differs in some important respects from generation of secretory granules (Bonanomi et al., 2006). Most vesicles transported from the neuronal cell body down the axon are actually tubulovesicular structures of 50nm diameter and variable length (Tsukita & Ishikawa, 1980), rather than the typical 50nm synaptic vesicle profiles. These observations as well as biochemical evidence suggest that not all synaptic vesicle components are transported in the same vesicle. Instead, these components might be transported as synaptic vesicle precursors (Szodorai, et al., 2009), which may not be competent to serve as synaptic vesicles. The current evidence suggests that some or all functionally competent synaptic vesicles are locally assembled at presynaptic terminals following an intermediate exocytosis and endocytosis event (see below). Subsequently, synaptic protein components are reconstituted as complete synaptic vesicles in the early endosome compartment. Consistent with this, synaptic vesicles are typically recycled many times and locally filled with neurotransmitter for reuse within minutes. In contrast, neuropeptide secretory granules can be used only once for secretion (Morris & Schmid, 1995). However, neurotransmitter vesicles containing noradrenalin are filled with transmitter during transport, leading to their characteristic dense core in electron micrographs. As a result, there may be some heterogeneity in synaptic vesicle biogenesis.

Once secretory vesicles are loaded with their cargo, they pinch off from the TGN and are actively transported to the specific subcellular site, where they fuse with the plasma membrane and release their cargoes. These sites may be quite far from the site of packaging and processing in the Golgi complex. In neurons for example, secretory vesicles carrying neuropeptides from the cell body (where peptides are synthesized and packaged into secretory vesicles) are transported down the axon to the presynaptic terminals, which in some neurons can be a meter or more away. Secretory vesicles are transported to sites of release through the action of microtubule-based motor proteins by processes collectively known as fast axonal transport (see Ch. 8).

As secretory vesicles mature, many secretory polypeptides undergo post-translational modifications. Many hormones and neuropeptides as well as hydrolytic enzymes are synthesized as inactive polypeptide precursors that need to undergo proteolysis to become active. This maturation process usually starts in the TGN and continues in the secretory vesicles, but may be completed in the extracellular space soon after exocytosis takes place in some cases. The maturation process for neuropeptides is described in detail in Chapter 20.

The fates of different secretory vesicles once they reach the plasma membrane vary. Those carrying cargo to be constitutively secreted will fuse with the plasma membrane once they arrive at their destination. In contrast, those carrying material to be secreted via regulated exocytosis pathways remain near the plasma membrane without fusing until a signal arrives that triggers vesicle fusion with the plasma membrane.

THE ENDOCYTIC PATHWAY PLAYS MULTIPLE ROLES IN CELLS OF THE NERVOUS SYSTEM

Most pathways in the endocytic system are shared with cells in general, but a special case exists in synaptic vesicle cycling, which is unique to neurons and a keystone in neuronal function. Two categories will be considered here: endocytic processes important for degradation of macromolecules and uptake of nutrients, and constitutive and receptor-mediated endocytosis. Synaptic vesicle cycling will then be considered separately and in greater detail.

Endocytosis for degradation of macromolecules and uptake of nutrients involves phagocytosis, pinocytosis and autophagy

The cellular processes by which macromolecules, particulate substances and even other cells may be taken up in a regulated fashion represent important aspects of the endocytic pathway (Conner & Schmid, 2003). These processes may also be important for invasion of the nervous system by viral vectors. There are three general mechanisms for the uptake of extracellular and intracellular materials: phagocytosis for engulfing macromolecules, particles and cells or viruses; pinocytosis for uptake of smaller molecules and fluids; and autophagy for degradation of intracellular organelles and aggregates.

Phagocytosis involves the uptake of large materials such as microorganisms or dead cells via large vesicles named phagosomes. Phagocytosis of large particles was developed early in the evolution; unicellular organisms use this type of endocytosis as a way to get nutrients, and the phenomenon is most familiar in macrophages. In multicellular organisms, phagocytosis has been developed as a defensive mechanism rather than for feeding purposes and is largely carried out by specialized cells in mammalian tissues. Three different white blood cells commonly exhibit phagocytosis in mammals: macrophages, neutrophils and dendritic cells. These different cell types posses a unique and complex function: they protect us from infections by phagocytosing the invading agents and they also take care of dead or senescent cells throughout the organism. In the nervous system, phagocytosis is normally conducted only by a specific type of glial cell: microglia, which are a specialized type of macrophage (Graeber, 2010). The function of these cells in the normal healthy brain is not well understood, but when these cells detect brain damage they proliferate, migrate to the site of injury or disease, turn on their macrophage capabilities, and engulf pathogens and

cellular debris (see Chs. 1, 33). In addition, vascular macrophages and related cells may on occasion be seen in nervous tissue, particularly in response to disease or damage.

Material to be internalized by phagocytosis is wrapped up by a special portion of the plasma membrane that invaginates first and then pinches off to form the phagosome, an endocytic vesicle that carries the phagocytosed material. The usual vesicle diameter size of phagosomes is >200 nm. Phagosomes eventually fuse with lysosomes; after a digestion process the metabolized products are released into the cytosol to be consumed as nutrients by the organism. Phagocytosis is a tightly regulated process that is initiated by a specific signal at the plasma membrane that is transmitted to the cell interior, which in turn initiates and regulates the phagocytosis process (Conner & Schmid, 2003). There are different signaling initiators to triggers phagocytosis; the best characterized are antibodies. Antibodies bind specifically to the surface of the invader organism, leaving the Fc region of the antibodies exposed to the exterior. These antibody Fc regions are recognized by specific surface receptors on the macrophages and neutrophils (chapter 33), which turn on the phagocytosis machinery. Negative charges on the cell surface of dead cells or cell debris can also trigger phagocytosis. There are also inhibitory signals displayed on the surface of living cells that prevent the activation of the phagocytosis pathway. One practical use of phagocytosis is for tracing neuronal pathways after they are labeled with specific markers like Fluorogold or horseradish peroxidase.

Pinocytosis involves the internalization of liquids and solutes via small pinocytic vesicles around 40–100 nm in diameter (Conner & Schmid, 2003). Unlike phagocytosis, pinocytosis is a constitutive process in almost all eukaryotic cells. Cells need to internalize fluids and solutes via small pinocytic vesicles, usually clathrin-coated vesicles around 40 nm in diameter. After passage through a sorting endosome, many of these small membrane vesicles return to the plasma membrane by exocytosis in non-growing or stationary cells. Cells must keep endocytosis and exocytosis processes tightly balanced; otherwise, in time the cell would run out of plasma membrane. Under normal circumstances, the volume and area of the cell in a stationary phase does not change during the endocytic–exocytic events even when rates of exocytosis become quite high, as observed in the presynaptic terminal. The two linked processes should then be viewed as a continuous endocytic–exocytic cellular cycle. The rate and extent of membrane internalization by pinocytosis varies among cell types. Some are extremely large, such as secretory cells and macrophages, one of which may internalize as much as a quarter of its volume in one hour.

Caveolae represent membrane invaginations of cholesterol- and sphingolipid-rich membrane domains. They are thought to mediate the transport of serum proteins into endothelial cells for transcellular transfer across the endothelium and may play a role in signaling pathways including nitrous oxide (NO) (Conner & Schmid, 2003). The major protein described in association with caveolae is caveolin, although its functions are not fully understood. Curiously, caveolin-null mice are phenotypically normal although subtle changes in specific cell types can be seen. Caveolae are particularly abundant on endothelial cells but can be detected on most cell types. They are not very abundant in neurons but may be important in NO signaling from endothelial cells of the brain.

The bulk of pinocytosis in the nervous system is mediated by clathrin-mediated endocytosis (CME) (Conner & Schmid, 2003,) and this is the best-characterized pathway. Pinocytosis through CME is responsible for uptake of essential nutrients such as cholesterol bound to low-density lipoprotein (LDL) and transferrin, but also plays a role in regulating the levels of membrane pumps and channels in neurons. Finally, CME is critical for normal synaptic vesicle recycling. Other pinocytotic pathways also exist that do not depend on either caveolae or clathrin, although these are not well defined (Conner & Schmid, 2003). Specific receptors continue to be internalized in the absence of clathrin or caveolin, and these pathways can be monitored by following glycosyl phosphatidylinositol (GPI)-anchored proteins. Non-clathrin, non-caveolin pathways may also be responsible for the reuptake of membrane in neuroendocrine cells after stimulated secretion. Some, but not all, of these pathways appear to require dynamin.

Autophagy involves the disposal of the obsolete part of the cells, such as mitochondria or SER. Three categories of autophagy have been described in cells (Cuervo, 2004) and all can be seen in the nervous system. Macroautophagy is typically accomplished by envelopment of organelles with an intracellular membrane known as an isolating membrane, eventually forming a double membrane structure known as an autophagosome (Yang & Klionsky, 2010). The engulfment isolates the organelle from the cytoplasm and the compartment may then be acidified. Eventually, the autophagosome fuses or is engulfed by a lysosome for degradation. Macroautophagy is induced by stress and may be seen following nerve or axonal damage. For example, resorption of myelin by Schwann cells in peripheral demyelination or wallerian degeneration is a dramatic example of macroautophagy (Wong & Cuervo, 2010).

Microautophagy is a constitutive pathway that is normally responsible for turnover of organelles (Cuervo, 2004). Unlike macroautophagy, there is no initial isolating membrane. Instead, the organelle fuses or is directly engulfed by the lysosome and degraded. This is an important pathway for recycling amino acids and other biological building blocks. Microautophagy is a major pathway available for degradation of insoluble aggregates and may be neuroprotective. Autophagic structures appear prominent in Parkinson's and Huntington's disease and amyotrophic lateral sclerosis, but the extent to which this is a neuroprotective pathway remains unclear (Wong & Cuervo, 2010). A combination of micro- and macroautophagy plays a key role in type II/nonapoptotic programmed cell death, a major cell death type during nervous system development. In type II programmed cell death, organelles are isolated and degraded, but the cytoskeleton is spared (Wong & Cuervo, 2010).

Chaperone-mediated autophagy is a mechanism for importing cytoplasmic proteins into lysosomes for degradation (Wong & Cuervo, 2010). This pathway has only been documented in mammalian cells to date and its cellular functions are not well defined. This process is analogous in some respects to the ubiquitin/proteasome pathway. Proteins

targeted for degradation are tagged with a particular KFERQ peptide motif, which is recognized by a chaperone. The chaperone–protein complex binds to a specific lysosomal membrane receptor for import into the lysosome and degradation. Autophagic profiles are common features of the aging brain and include some of the characteristic histological features of the aging brain (lipofuscin, ceroid, multivesicular bodies, etc.) (Wong & Cuervo, 2010).

Retrieval of membrane components in the secretory pathway through receptor-mediated endocytosis (RME) is a clathrin-coat-dependent process

The clathrin coat provides stability to the vesicle core and allows uptake of specific membrane proteins for reuse or degradation. RME shows a remarkable degree of specificity, allowing cells to internalize selected molecules with astonishing efficiency, independent of their extracellular concentration.

Extracellular ligands (hormones, neurotrophins, carrier proteins, adhesion molecules, small molecules, etc.) bind to specific transmembrane receptors. This binding of specific ligand induces the concentration of the receptor in coated pits and internalization via clathrin-coated vesicles. One of the best-studied and characterized examples of RME is the internalization of cholesterol by mammalian cells (Herz & Bock, 2002). In the nervous system, there are a plethora of different membrane receptors that bind extracellular molecules, the best examples studied include neurotrophins, hormones and other cell modulators. This type of clathrin-mediated endocytosis is an amazingly efficient process, capable of concentrating receptors several hundredfold and taking up large quantities of specific molecules. As a result, even a very dilute extracellular ligand can be internalized without taking up a correspondingly large amount of extracellular fluid.

In the CNS, neurons are highly dependent on a continual supply of small polypeptide growth factors with neurotrophic activity (see Ch. 29). This process requires the binding of the secreted polypeptides to specific transmembrane receptors with an intrinsic tyrosine kinase activity on their cytoplasmic domains (Fig. 7-7). Upon binding of peptide growth factor, these receptor tyrosine kinases (RTKs) (Ch. 26) activate their intrinsic tyrosine kinase activity and induce RME (Huang & Reichardt, 2003). The sequence of events typically follows a pattern of ligand binding, followed by RTK dimerization and activation. Once activated, RTKs autophosphorylate on specific cytoplasmic tyrosines and recruit proteins containing Src homology II (SH2) and phosphotyrosine-binding domains. Among these are linking proteins and adaptor proteins for assembly of a clathrin coat. The signaling begins with membrane internalization and continues as the RTKs and associated molecules are packaged and returned to the cell body (Cosker et al., 2008; Howe & Mobley, 2004).

Cells control the amount of available receptors on their surface as well as the level of extracellular ligand by regulating rates of RME and insertion of new receptors at the plasma membrane. The internalization of activated receptors through clathrin-coated pits and their translocation to endosomes lowers the number of receptors in the plasma membrane, a process known as downregulation. Cells reduce or attenuate the receptor-mediated intracellular signaling via this downregulation. Alternatively, removing the extracellular ligands via RME will also attenuate intracellular signaling. Thus, intracellular signaling and endocytosis are tightly linked, although many questions remain to be answered (Howe & Mobley, 2004).

Electron microscopic studies on cells stimulated simultaneously with different labeled ligands indicate that RME can concentrate up to 1,000 different transmembrane receptors in a single clathrin-coated pit. In the canonical model of clathrin-mediated endocytosis, these clathrin-coated pits of the plasma membrane bud off to form clathrin-coated vesicles. These coated vesicles rapidly lose their clathrin coats through the action of a chaperone from the Hsp70 family, an event that facilitates local fusion with early endosomes (EEs). The various endocytic vesicles fuse with the membrane of the same EEs soon after shedding their clathrin coats. However, the fate of various ligand-receptor complexes varies according to their physiological role and cellular demand. In non-neuronal cells, electron microscopy studies reveal very complex endosomal compartments composed of a highly dynamic array of heterogeneous tubulovesicular membrane structures extending from close vicinity to the plasma membrane to the cell interior, reaching the boundaries of the Golgi apparatus. Presynaptic terminals have similar endosomal systems, albeit less extensive (Murthy & De Camilli, 2003; Sudhof, 2004).

The endocytic pathway also contains functionally and physically discrete compartments (Mellman, 1996). EEs are slightly acidic (pH 6.0–6.8), which induces the dissociation of many, but not all, receptor–ligand complexes. Free receptors may then concentrate in the tubular portion of EEs, where they bud off to form recycling vesicles. These vesicles transport receptors that are directly or indirectly returned to the plasma membrane. Free ligands are typically kept in the vesicular portion of the EEs. Depending on the ligand and the cell, ligands that provide essential cellular components (transferrin, etc.) can be actively transported to the perinuclear region of the cell, where they fuse with late endosomes (LEs) and the ligand is delivered to the appropriate compartment. Alternatively, once the ligand is transported to the perinuclear region of the cell and LE, the LE will fuse with lysosomes where ligands may be degraded by the lysosomal enzymes in an acidic environment (pH 5.0). Lysosomal enzymes are transported from the TGN to the LEs and lysosomes via the same type of clathrin-coated vesicles. Thus, clathrin-coated vesicles generate vesicular structures designed to fuse with endosomes independent of their origin in either the plasma membrane or the TGN.

EEs with their slightly reduced pH are sorting compartments responsible for the dissociation and sorting of the ligands to the LEs and lysosomes, as well as for directing receptors back to the plasma membrane for reuse. Therefore, EEs act as the first sorting station in the endocytic pathway. EEs also play an important role in synaptic vesicle recycling (see below). LEs serve to concentrate and sort endocytosed material further, including for delivery to lysosomes where both endogenous and exogenous macromolecules are digested.

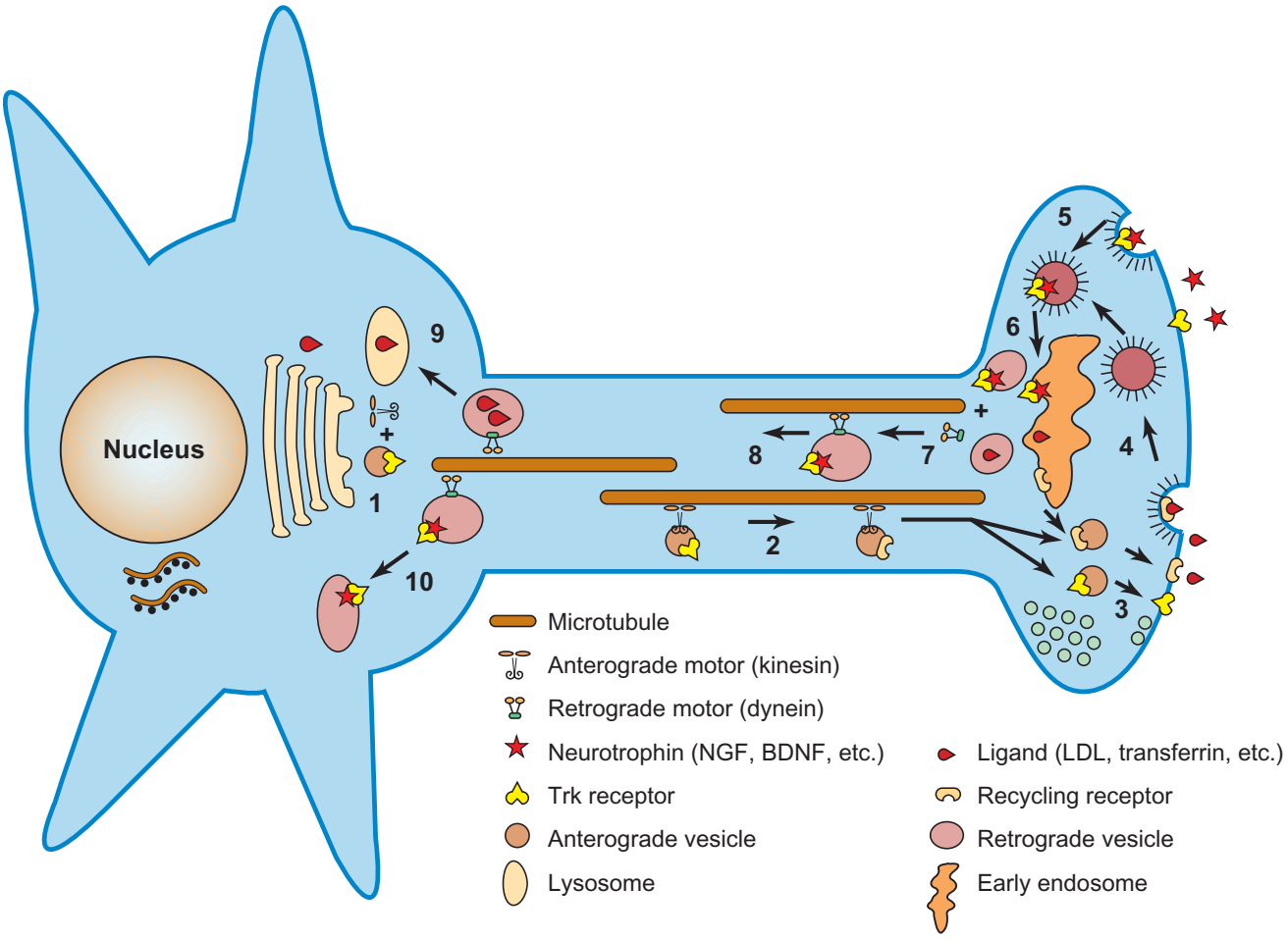

FIGURE 7-7 **Receptor-mediated endocytosis.** Many kinds of extracellular polypeptides and ligands (including hormones, carrier proteins, adhesion molecules, neurotrophins, etc.) are imported into the cell with a high degree of specificity via a special type of clathrin-mediated endocytosis. A high degree of molecular specificity is achieved through binding of ligands to specific receptors localized within discrete domains of the plasma membrane. The unique dependence on membrane receptors for the internalization of these different extracellular molecules led to the name 'receptor-mediated endocytosis' (RME). RME ensures the internalization of selected molecules, independent of the extracellular concentration of the ligand. As a result, even very dilute extracellular ligands can be internalized without taking up a correspondingly large quantity of extracellular fluid. One of the best-studied receptor-mediated endocytosis pathways is the internalization of neurotrophins along with their specific receptor tyrosine kinase receptors (RTK). The internalization of the neurotrophins and growth factors is achieved through a well-defined sequence of events: *(1)* RTKs are synthesized and packaged in the Golgi. Anterograde motor proteins (i.e., conventional kinesin) bind the newly formed vesicles, then *(2)* these vesicles are transported along microtubules to the appropriate membrane domain (i.e., presynaptic terminals), where *(3)* the receptors are delivered and inserted into the plasma membrane. This typically occurs through a form of targeted constitutive secretion. *(4)* Binding of a suitable ligand to a typical recycling receptor like the LDL receptor or the transferrin receptor leads to formation of a coated pit and coated vesicle. The coat is removed and the interior of the endocytosed vesicle is acidified, leading to dissociation of receptor and ligand, followed by fusion with an early endosome (EE). *(5)* Alternatively, stimulation of cells with a neurotrophin or a growth factor results in the clustering of the growth factor–RTK complexes into clathrin-coated pits. Ligand–RTK complexes are internalized by regulated clathrin-mediated endocytosis but the receptor ligand complex does not dissociate. Clathrin-coated vesicles carrying the growth factor–RTK complexes shed their clathrin coats soon after internalization, before being translocated and fused with EEs. *(6)* In the EE, receptors are sorted. If ligands and receptors are dissociated by the slightly acidic pH in the EEs, the receptor is typically recycled back to the plasma membrane to participate in a new cycle of endocytosis. *(7)* The ligands are then packaged into a vesicle for return to the cell body. However, neurotrophins remain bound to their RTKs and are sorted into a specialized retrograde endosome that may continue to signal. At this stage, the retrograde motor protein dynein is added to the vesicle. *(8)* Retrograde vesicles containing ligands or growth factor–RTK complexes are actively transported and returned to the neuronal cell body by retrograde axonal transport (see Ch. 8), where *(9)* ligand-containing vesicles and worn-out membrane proteins are fused with lysosomes for eventual degradation and recycling at the end of the journey. *(10)* Vesicles containing neurotrophin–RTK complexes continue to signal for a period of time before degradation, leading to changes in gene expression. In this way, endocytosis allows for communication of signaling events between the neuronal cell body and distant regions of the neuron interacting with their targets (i.e., a muscle cell or another neuron).

Ligand–receptor complexes that do not dissociate in the EEs have different fates. In some cells, they may be returned to the same plasma membrane compartment from which they originated, whereas in polarized cells such as endothelial cells or astrocytes they can be moved to a different plasma membrane domain, resulting in transcytosis. In other cases, the complexes go to LEs and lysosomes for degradation. In neurons, these vesicles may serve as signaling organelles that are transported from the EEs back to the cell body, where they influence gene expression (Cosker et al., 2008; Howe & Mobley, 2004).

Details of the mechanisms by which endocytosed material moves from the early to the late and lysosomal compartment are still poorly understood. However, portions of the EEs tubulovesicular structures may be actively transported along microtubules towards the perinuclear region of the cell in both neurons and non-neuronal cells. These endosomes on the move may enclose invaginated membranes and also internally bud off vesicles. For that reason, these complex structures are called multivesicular bodies (MVBs) (Weible & Hendry, 2004). Material returning by retrograde axonal transport to the neuronal cell body includes many MVBs (Tsukita & Ishikawa, 1980). The eventual fate of these structures may vary. Some MVBs may fuse with LEs or fuse with each other to form LEs. These LEs eventually become lysosomes, a process that requires the fusion of hydrolase-containing vesicles coming from the TGN and acidification of their lumens. In MVBs we can find internalized membrane proteins on their way to be degraded, as well as others that have to be recycled and possibly some that are actively signaling organelles.

SYNAPTIC VESICLE TRAFFICKING IS A SPECIALIZED FORM OF REGULATED SECRETION AND RECYCLING OPTIMIZED FOR SPEED AND EFFICIENCY

Some neurons can fire several hundred times per second, secreting neurotransmitter with each round of firing. From this observation, it becomes clear that synapses are remarkable secretion machines destined to undergo millions of repeated exocytic cycles in their lifetime. So how does this process work so fast and so efficiently? Answers have gradually emerged from the work of many different laboratories and model systems (Murthy & De Camilli, 2003; Sudhof, 2004). An exhaustive description is beyond the scope of this chapter, but a summary of key events and specializations of the synaptic vesicle cycle is useful. This section will describe how synaptic transmission is optimized spatially and biochemically.

The organization of the presynaptic terminal is one important element for optimization of secretion and recycling

The presynaptic terminal is compact so that vesicles may rapidly move from one organelle compartment to another. Unlike endocrine secretion, where membrane fusion occurs randomly on a relatively large membrane area, synaptic vesicles are secreted only at active zones after docking has taken place. The process of docking means that vesicles are primed for membrane fusion in response to highly localized changes in Ca^{2+}. The ability to dock at an active zone is unique to synaptic vesicles. The peptidergic, dense core vesicles also found in most presynaptic terminals are released through unspecialized membrane regions, and the kinetics of peptide secretion is similar to release of hormones from endocrine cells. The composition of synaptic vesicles, the active zone and many of the components needed for docking have been identified (Murthy & De Camilli, 2003; Sudhof, 2004) (see Table 7-2 for a list of proteins and putative functions).

One characteristic of regulated exocytosis is the ability to store secretory vesicles in a reserve pool for utilization upon stimulation. In the presynaptic terminal, this principle is expanded to define multiple pools of synaptic vesicles: a readily releasable pool, a recycled synaptic vesicle pool and a larger reserve pool. This reserve pool assures that neurotransmitter-containing vesicles are available for release in response to even the highest physiological demands. Neurons can fire so many times per minute because synaptic vesicles from the readily releasable pool at a given synapse undergo exocytosis in response to a single action potential. Those vesicles have been primed by docking at the active zone and are therefore ready for exocytosis upon arrival of an action potential. However, for the synapse to respond rapidly and repeatedly under heavy physiological demand, these exocytosed vesicles must be rapidly replaced. This is accomplished first from the recycled pool of vesicles and, as the demand increases, from the reserve pool. To be recycled, synaptic vesicles must be reloaded quickly after they release their contents. The sequence of events that is triggered by neurotransmitter exocytosis is known as the synaptic vesicle cycle (Murthy & De Camilli, 2003; Sudhof, 2004) (Fig. 7-8). In neurons, an action potential triggers a highly localized rise of synaptic intracellular Ca^{2+} through opening of voltage-gated Ca^{2+} channels closely apposed to the active zones. These Ca^{2+} ions bind proteins that serve as Ca^{2+} sensors, among them specific synaptotagmins, which in turn trigger the vesicular membrane fusion with the plasma membrane for docked synaptic vesicles (Pang & Sudhof, 2010).

In a simplistic model, the exocytosis step of neurotransmission takes place in at least three major different steps

First, synaptic vesicles dock to a special region of the plasma membrane at the synapse, the active zone. Once docked, synaptic vesicle proteins (v-SNAREs) and plasma membrane proteins (t-SNAREs) become closely apposed but not activated. Second, after vesicle docking and prior to a vesicle fusion event, these vesicles must undergo an ATP-dependent 'priming' process requiring the activity of NSF (NEM-sensitive factor) and phosphoinositide-transfer proteins. This priming event prepares synaptic vesicles to fuse their membranes with the synaptic plasma membrane within

TABLE 7-2 A Glossary of Proteins in the Synapse

1. Synaptic vesicle proteins	
Cysteine string protein (CSP)	Peripheral membrane protein that is palmitoylated on >10 cysteines. May have a role in Ca^{2+} sensitivity of exocytosis.
Neurotransmitter transporters	There are probably at least 5 types of transport protein specific for glutamate, acetylcholine, catecholamines, glycine/GABA and ATP. The type of transporter contributes to determining the transmitter specificity of a synapse.
Rab and Ra1 proteins	Rab3A, Rab3C, Rab5, Rab7 and Ra1. Since Rab proteins cycle between cytosolic and membrane-bound forms, not all synaptic vesicles contain all Rab proteins at the same time. Rab proteins regulate docking and fusion processes.
Rabphilin-3A	Peripheral membrane protein that binds to Rab3A and Rab3C as a function of GTP, is substrate for multiple protein kinases and contains two C-terminal C2 domains that may bind Ca^{2+}.
Secretory carrier membrane proteins (SCAMPs)	Ubiquitous integral membrane proteins of secretory and transport vesicles of unknown function.
SV2s	Highly glycosylated proteins with at least three isoforms (SV2A, B and C) containing 12 transmembrane regions and homology to bacterial and eukaryotic transporters. May help regulate Ca^{2+} levels.
Synapsins Ia, Ib, IIa and IIb	Monotopic membrane proteins with common N-terminal domains, with phosphorylation sites for CaMKI and PKA but diverge C-terminally. Synapsins Ia/b contain C-terminal phosphorylation sites for CaMKII and CDK 5. Interact with microfilaments, neurofilaments, microtubules, SH3 domains, calmodulin and annexin VI *in vitro*.
Synaptobrevins (VAMPs)	Small-membrane proteins that are cleaved by tetanus toxin and by botulinum toxins B, D, F and G.
Synaptogyrin	Polytopic membrane protein that is tyrosine-phosphorylated. Function unknown.
Synaptophysins	Polytopic membrane proteins, including synaptoporin, that are tyrosine-phosphorylated and bind to synaptobrevins. May regulate SNARE function.
Synaptotagmins	Membrane proteins with at least 15 isoforms that contain C2 domains; bind Ca^{2+} and phospholipids; and interact with neurexins, AP2 and syntaxins. Synaptotagmins 1 and 2 may function as Ca^{2+} sensors in fast Ca^{2+}-dependent neurotransmitter release.
Transport proteins (channels) for chloride and zinc	Components of synaptic vesicles to mediate the chloride flux for glutamate uptake and zinc uptake in most synaptic vesicles. Zinc transporter is homologous to endosomal and plasma membrane zinc transporters; chloride transporters remain to be identified.
Vacuolar proton pump	Protein complex of more than 12 subunits. Constitutes the largest component of synaptic vesicles and establishes electrochemical gradient for neurotransmitter uptake.
2. Proteins that associate with synaptic vesicles or their precursors	
Amphiphysin	Nerve-terminal protein that associates with synaptic vesicles probably via AP2 bound to synaptotagmin. May function in endocytosis.
AP2 and clathrin	AP2 is a protein complex that binds to a specific receptor on synaptic vesicles and plasma membranes to trigger assembly of clathrin for endocytosis.
Ca^{2+}, calmodulin-dependent protein kinases I and II (CaMKI and CaMKII)	May transiently associate with synaptic vesicles to phosphorylate synapsins and rabphilin-3A. May regulate various steps in neurotransmitter release.
Dynamin-1	GTPase required for endocytosis that is phosphorylated by protein kinase C and dephosphorylated by calcineurin upon membrane depolarization and binds to AP2. Important for budding and fusion pore closure.
Dynein	Motor protein mediating retrograde fast axonal transport (see Ch. 8).
Kinesins	Motor proteins for microtubule-based synaptic vesicle transport (see Ch. 8).
GDP-dissociation inhibitors (GDIs)	Bind isoprenylated Rab proteins in the GDP-bound form, resulting in a cytoplasmic complex.
MSS4	Ubiquitous protein that tightly binds to a subgroup of Rab proteins, including Rab1, Rab3 and Rab8. Function unknown.
pp60src	Tyrosine kinase that phosphorylates synaptophysin and synaptogyrin.
3. Presynaptic plasma membrane proteins	
Munc13s	Mammalian homologs of the *C. elegans* unc-13 gene that is essential for exocytosis. Binds phorbol esters but is not a protein kinase. A component of active zones that interact with RIM, syntaxin and other proteins.
Neurexins	Cell surface proteins with more than 1,000 isoforms generated by alternative splicing from three genes. Neurexins include one of the receptors for α_1-latrotoxin and may function in cell–cell recognition between neurons.

(Continued)

TABLE 7-2 (Continued)

SNAP-25	Palmitoylated peripheral membrane protein that is cleaved by botulinum toxins A and E and binds to syntaxins.
Syntaxins	Ubiquitous membrane proteins that are cleaved by botulinum toxin C1 and bind to synaptotagmins, SNAP-25, synaptobrevins, complexins, munc13s, SNAPs, Ca^{2+} channels and munc18s.
Voltage-gated Ca^{2+} channels	Mediate Ca^{2+} influx for neurotransmitter release at the active zone.
RIM	Binds to Rab3 in a GTP-dependent manner and may mediate Rab3 action in regulating fusion.
4. Proteins that reversibly associate with synaptic plasma membrane proteins	
Munc18s	Mammalian homologs of the *C. elegans unc-18* gene and the *sec-1*, *sly-1* and *slp-1* products of yeast. Bind tightly to syntaxins.
N-ethylmaleimide-sensitive factor (NSF)	Trimeric ATPase required for *in vitro* membrane fusion during vesicular transport. Probably function as chaperones in synaptic vesicle recycling.
α/β/γ-SNAPs	Soluble NSF-attachment proteins required to recruit NSF to membranes in an ATP-dependent manner.

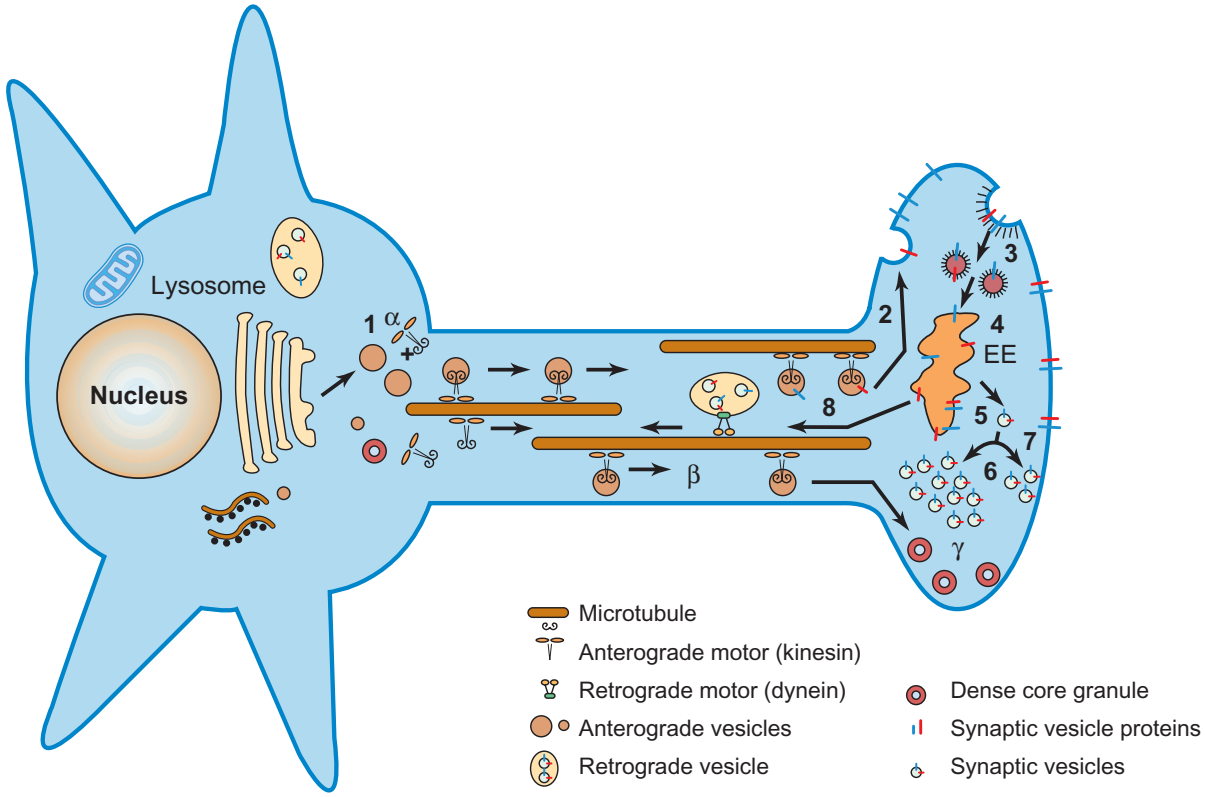

FIGURE 7-8 **The life cycle of synaptic vesicles.** As with other secretory vesicles, *(1)* membrane components of synaptic vesicles are synthesized in the cell body, packed into membrane-bounded transport vesicles, combined with kinesin motor proteins and actively transported down the axon to the synaptic plasma membrane via the constitutive secretory pathway. However, not all synaptic vesicle proteins (*red and blue*) are packaged together, so the synaptic vesicle requires additional steps for reconstitution. Neurons typically release neuropeptides, as well as standard neurotransmitters. These are prepared as illustrated in Figure 9-7 for more typical regulated secretory vesicles (α) because their contents must be synthesized in the cell body. *(2)* Once a synaptic vesicle precursor has been transported to the presynaptic terminal, it fuses with the plasma membrane constitutively. Dense core granules are similarly transported down the axon (β). They mature during transport, but are otherwise competent for regulated secretion. *(3)* Synaptic vesicle membrane proteins are then gathered efficiently through receptor-mediated endocytosis in a clathrin-mediated process. (4) Soon after the endocytosed vesicle pinches off, it sheds its clathrin coat and is transported to the early endosomes (EE) where the components for a synaptic vesicle are sorted. *(5)* They then bud off from the EE to form empty synaptic vesicles. These are rapidly loaded with neurotransmitter via active transport across the membrane. *(6)* At this stage, the synaptic vesicle is translocated either to the large reserve pool of synaptic vesicles or back to the plasma membrane, where it docks again to the synaptic active zone. Vesicle exocytosis is a process that requires an ATP-dependent priming process (see text) prior to membrane fusion triggered by Ca^{2+} influx in response to an action potential. *(7)* After release of neurotransmitter, synaptic vesicle membrane components may be recycled by repeating steps 3–6. This recycling can occur very rapidly. *(8)* Eventually, some synaptic vesicle components will be repackaged into retrograde vesicles for return to the cell body and degradation. The dense core granules (γ) are not associated with active zones or other specialized structures. These peptidergic vesicles typically require higher levels of intracellular Ca^{2+} and have a much slower rate of release.

milliseconds after an action potential has reached the synapse. The synaptic vesicle protein Munc13 and the presynaptic protein RIM, localized to the active zone, play critical roles in the priming process. Third, when an action potential reaches the synapse, voltage-gated Ca^{2+} channels open and Ca^{2+} ions flow into the synaptic terminal, raising the local intracellular Ca^{2+} concentration in the immediate vicinity of the active zone and docked vesicles. These free Ca^{2+} ions bind Ca^{2+}-binding proteins, including synaptotagmins, which in turn initiate vesicular membrane fusion with the plasma membrane through conformational changes in both vesicle and plasma membrane proteins.

As noted above, functionally competent synaptic vesicles are not typically generated at the level of the TGN. Instead, they are assembled from endocytosed material retrieved from the synaptic plasma membrane. Synaptic vesicle proteins and plasma membrane lipids are synthesized in the endoplasmic reticulum and modified in the Golgi apparatus, where they are then packaged in secretory vesicle precursors. These synaptic precursors are delivered to the plasma membrane from the cell body by the constitutive secretory pathway. Synaptic vesicle proteins must be retrieved by clathrin-mediated synaptic vesicle endocytosis, a variant of RME with some neuron-specific components. Once the vesicle sheds its clathrin coat, the uncoated vesicle fuses with a local EE, where the synaptic vesicle proteins are sorted to form mature synaptic vesicles. The sorted proteins bud off from the EE as an empty synaptic vesicle, and neurotransmitter is then taken up into the mature synaptic vesicle in the nerve terminal. The loaded, fully functional synaptic vesicle is actively transported to the reserve pool of vesicles, where it waits until it is mobilized to the active zone of the synaptic terminal, eventually docking at the presynaptic plasma membrane. After being discharged, synaptic vesicle membrane along with the protein components are retrieved by regulated endocytosis and recycled for additional rounds of secretion.

Many years have passed since the concept of synaptic vesicle recycling was introduced in the early 1970s, but details of the synaptic vesicle cycle continue to be a matter for investigation and debate

The role of clathrin-coated vesicles in the rapid form of endocytosis that characterizes synaptic transmission has been well established as a mechanism for synaptic vesicle recycling (Morris & Schmid, 1995), but this classical model was difficult to reconcile with some observations. In recent years, evidence has mounted to support the existence of other pathways that diverge from the classical picture. In particular, membrane fusion may not always be complete. In these cases, the clathrin-mediated endocytosis and sorting endosome steps may be bypassed under certain circumstances. Although these models were originally proposed as an alternative to the classical model, most investigators now acknowledge that these pathways are not mutually exclusive.

In the classic model of synaptic vesicle recycling in nerve terminals, synaptic vesicles fuse completely with the plasma membrane and the integrated vesicle proteins move away from the active zone to adjacent membrane regions (Fig. 7-9A). In these regions, clathrin-mediated synaptic vesicle endocytosis takes place rapidly (within seconds) after neurotransmitter release (Morris & Schmid, 1995). The process starts with the formation of a clathrin-coated pit that invaginates toward the interior of the cell and pinches off, primarily through the activity of dynamins, to form a clathrin-coated vesicle (Shupliakov & Brodin, 2010). Clathrin-coated vesicles are transient organelles that rapidly shed their coats in an ATP/chaperone-dependent process. Once uncoated, the recycled vesicle fuses with a local EE for reconstitution as a synaptic vesicle. Subsequently, the recycled synaptic vesicle is filled with neurotransmitter and it returns to the release site ready for use. This may be the normal pathway when neurotransmitter release rates are modest. Clathrin/EE-based pathways become essential when synaptic proteins have been incorporated into the presynaptic plasma membrane.

However, an alternative pathway that bypasses clathrin-mediated endocytosis and EEs appears to be available as well. This model of endocytosis, known as 'kiss and run' or its variant, 'kiss and stay', has attracted increasing interest in recent years (Murthy & De Camilli, 2003; Sudhof, 2004) (Fig. 7-9B). 'Kiss and run' has been directly demonstrated with dense-core granules in neuroendocrine cells (Breckenridge & Almers, 1987; Taraska & Almers, 2004), and this model helps explain some observations that are not readily accommodated by the classical pathway. The 'kiss and run' model proposes that neurotransmitters are released by a transient fusion pore, rather than by a complete fusion with integration of the synaptic vesicle components into the plasma membrane. Synaptic membrane proteins never lose their association, and the vesicle reforms when the pore closes. As a result, the empty vesicle can be refilled and reused without going through clathrin-mediated endocytosis and sorting in the EEs.

Recent experiments involving biochemically isolated rodent synapses (synaptosomes) provided evidence for the 'kiss-and-run' model in mammalian synapses (Serulle et al., 2007). However, direct demonstrations of the 'kiss-and-run' model in living mammalian synapses remains elusive (He & Wu, 2007; Murthy & De Camilli, 2003). However, there is sufficient evidence to suggest that there may be up to three types of endocytic pathways operating in the presynaptic terminal: the classic, clathrin-mediated endocytic pathway and two faster pathways that do not depend on clathrin coat assembly. In the first, known as 'kiss and stay,' the vesicles remain docked for refilling. In the second, referred to as 'kiss and run,' vesicles recycle locally after forming a transient pore and then releasing from the membrane. Consistent with the existence of one or both of these latter models, elegant studies done with *Drosophila* indicate that flies with mutations in endophilin, a key endocytic protein required for clathrin-mediated endocytosis, have synapses that contain very few vesicles. However, these synapses can sustain synaptic transmission at low frequencies, suggesting that multiple pathways are operating in the presynaptic terminal.

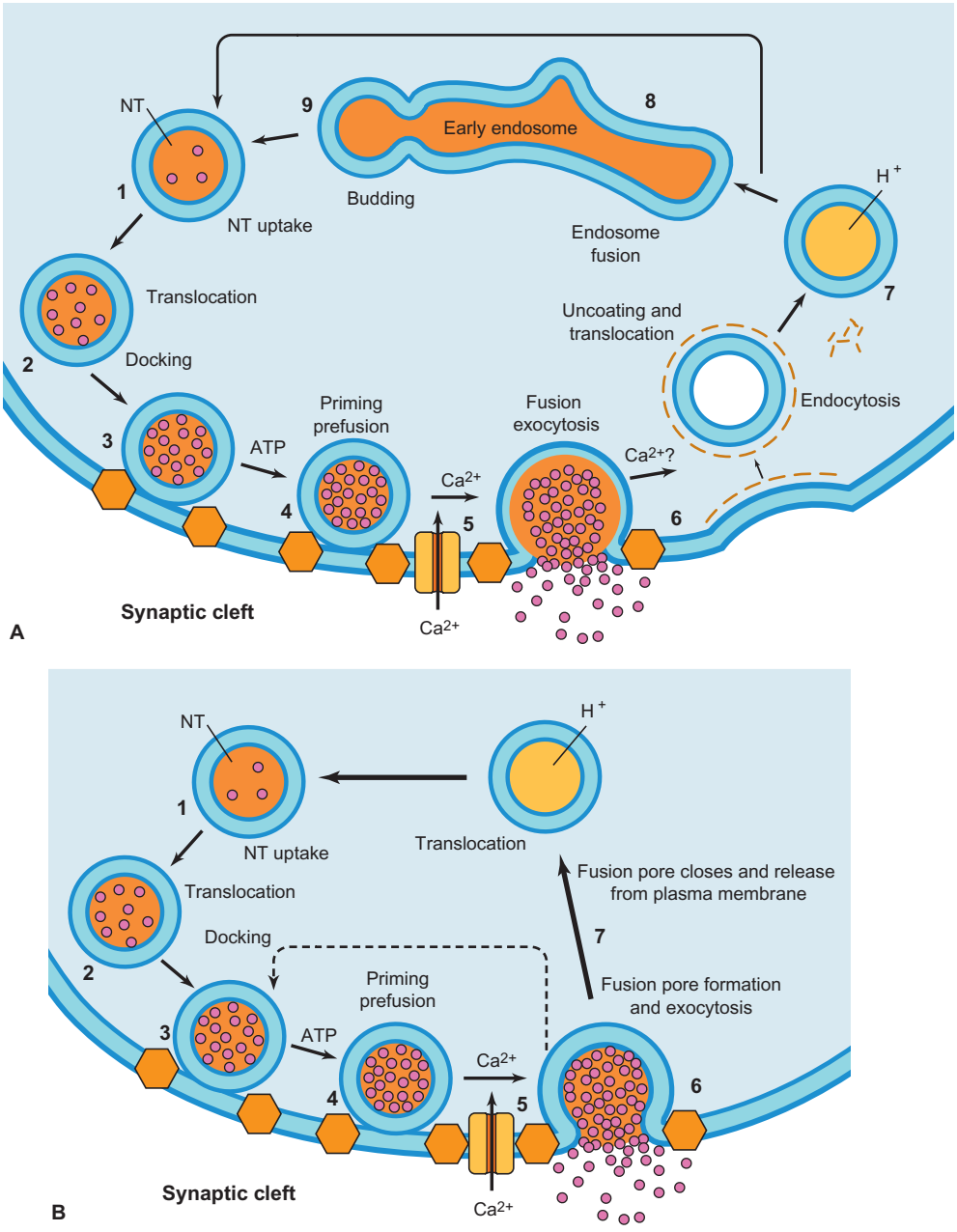

FIGURE 7-9 **Release of neurotransmitter from synaptic vesicles is rapid and highly specialized.** **(A)** The canonical pathway for neurotransmitter release involves a specific sequence of events. (*1.*) The first step in neurotransmitter release is to fill the synaptic vesicle (SV) with the appropriate neurotransmitter. This is accomplished by specific transporters in the SV membrane and is ATP-dependent. (2.) After filling, SVs may be moved to a reserve pool or to specialized regions of the presynaptic plasma membrane known as active zones (indicated by *orange hexagons*). (3.) SVs dock at the active zones where (*4*) they are primed for the fusion event. When an action potential arrives at the terminal (*5*), there is a local influx of Ca^{2+} through voltage-gated channels that triggers fusion of the SV with the plasma membrane and release of neurotransmitter. In the canonical pathway (*6*), SV membrane components are rapidly displaced from the active zone and gathered into coated pits for endocytosis. (*7.*) The resulting coated vesicle begins to acidify and the clathrin coat is removed by a chaperone. (*8.*) These endocytosed vesicles fuse with an early endosome for sorting and reconstitution, or may be directly refilled (*9*) with neurotransmitter for reuse. Although there is considerable evidence in support of this model, evidence exists for a more rapid alternative (Murthy & De Camilli, 2003; Sudhof, 2004). **(B)** Two variants of this mechanism have been proposed, called respectively ' kiss and run' (*solid arrows*) and 'kiss and stay' (*dashed arrow*). In both of these models, the initial steps of (*1*) filling the SV with neurotransmitter, (2) translocation to the active zone, (*3*) docking of the SV and (*4*) priming of the SV prior to fusion occur as in the canonical pathway. However, (*5*) influx of Ca^{2+} leads to the transient formation and rapid closure of a 'fusion pore' for release of neurotransmitter without integration of synaptic vesicle proteins into the plasma-membrane. (*6*) Recycling of synaptic vesicles proceeds by a less well-understood mechanism that does not involve clathrin, formation of a coated pit or vesicle, or sorting in an early endosome. Because the SV never loses its integrity (*7*), it may be released from the plasma membrane to reenter at step (1) or may remain docked at the active zone (3) where it is refilled and reprimed (4). These pathways and the classic pathway are not mutually exclusive and both kinds of release may occur in a presynaptic terminal.

Acknowledgments

The authors would like to thank Gregorio Gavier for help on figure design. Preparation of this chapter was supported in part by grants from the National Institute of Neurological Disease and Stroke to Drs. Brady and Morfini.

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