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MYASTHENIA GRAVIS AND MYASTHENIC DISORDERS

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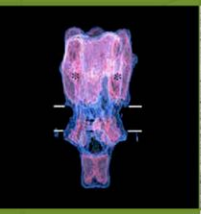

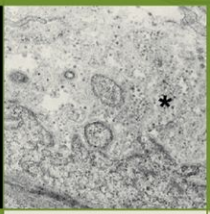

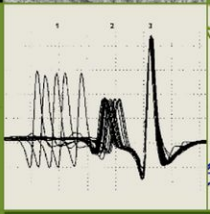

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MYASTHENIA GRAVIS AND MYASTHENIC DISORDERS

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This book is dedicated to patients affected by myasthenia gravis and myasthenic syndromes.

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Preface

The first edition of *Myasthenia Gravis and Myasthenic Disorders* was published more than a decade ago. Numerous advances in the field have prompted me to invite outstanding investigators and clinicians to contribute to the second edition.

The initial chapters review the anatomy and molecular architecture of the neuromuscular junction and the electrodiagnosis of defects of neuromuscular transmission. The choice of these topics was dictated by the belief that all myasthenic disorders arise from structural or functional alterations in one or more components of the neuromuscular junction and that detecting a defect in neuromuscular transmission is an essential first step in identifying and defining any myasthenic disorder.

In Chapter 3, Reinhard Hohlfeld, Hartmut Wekerle, and Alexander Marx describe important advances in the pathogenesis of MG. There is now irrefutable evidence that the MG thymus contains the complete cellular and molecular machinery for activating autoimmune T cells and B cells, the acetylcholine receptor (AChR) autoantigen, and antigen-presenting cells that efficiently present immunogenic epitopes of AChR recognized by autoreactive helper T cells. Recently, the autoimmune regulator AIRE has taken center stage in determining intrathymic expression of numerous self-proteins that form a "mirror image" of the entire repertoire of the body's self-antigens. The result is exposure of developing T cells to self-proteins of other tissues, including muscle. AIRE thus plays a crucial role in tolerance induction, and its malfunction leads to autoimmune disease and directly relates to the intrathymic pathogenesis of MG. On the other hand, the role of the thymus in thymoma-associated MG and in anti-AChR antibody-negative MG is still not well defined.

David Grob's observations on the manifestations and the clinical course of MG in more than 1,000 patients gleaned over 60 years are still valid. The revised Chapter 4 by Vladimir Luchanok and Henry Kaminski retains much of Grob's original work, even if the widespread use of immunotherapy today masks variations of disease severity and spontaneous remissions.

In Chapter 5, James Howard, Jr. elegantly reviews the clinical clues, the currently available test for diagnosis, and the differential diagnosis of MG, and compares the clinical and immunological features of the MuSK antibody-positive and the AChR antibody-positive myasthenias.

In Chapter 6, Daniel Drachman authoritatively summarizes the treatment of MG. The chapter includes up-to-date information on the use of CellCept, cyslosporine, tacrolimus, and methotrexate; instructions for patients taking immunomodulatory medications; and the use of rituximab in poorly controlled MuSK antibody-positive MG. The chapter also discusses high-dose cyclophosphamide therapy, the use of multiple agents to treat MG, and how the patient's condition and needs dictate the urgency of treatment.

In Chapter 7 on the Lambert-Eaton syndrome, Vern Juel and Donald Sanders point out that LEMS remains rare and is probably underdiagnosed. Although many patients encounter proximal leg weakness and fatigue as the most salient symptom, there is increasing appreciation of ocular and bulbar distributions of weakness. Though LEMS is largely a disease of adults, there are several contemporary reports of noncarcinomatous LEMS in children and as a transient neonatal disorder.

Chapter 8 by Andrew Engel, Xing-Ming-Shen, Kinji Ohno, and Steven Sine gives a full account of the current status of the congenital myasthenic disorders several of which have been described in the past decade. The CMS disease proteins now include not only subunits of the AChR and the ColQ component of acetylcholinesterase, but also choline acetyltransferase, β2-laminin, rapsyn, plectin, agrin, MuSK, Nav1.4, Dok-7, and GFPT1, and still more await discovery. Importantly, when correctly identified, most of the congenital myasthenic syndromes are amenable to therapy.

The companion Chapter 9 by Steven Sine and Andrew Engel describes how knowledge of the AChR increased during the past decade and dissects the structural and mechanistic consequences of

the kinetically significant mutations of AChR. By 1999, primary sequences of the AChR subunits were determined and sequences contributing to the agonist binding site, and the ion channel and its selectivity filter, were defined. Identification of mutations causing CMS combined with functional analyses disclosed additional functionally significant sites. Cryoelectron microscopy of the *Torpedo* AChR revealed a cylindrical silhouette, half extracellular and the rest intramembrane and intracellular. This, however, was not enough to reconstruct the 3D structure of the AChR subunits. After 1999, x-ray structural analyses of the molluscan ACh binding protein (AChBP) and subsequently of *Torpedo* AChR rationalized the finding of previous functional studies, revealed atomic scale interactions that stabilize AChR ligands, and facilitated molecular dynamics simulations revealing conformational changes triggered by agonist binding. Just recently, the atomic structure of an AChR ligand binding domain was solved with and without bound agonist. These studies, in turn, catalyzed further studies that have elucidated molecular events in the fleeting transitional state during which the receptor isomerizes from the closed to the open state and revealed an unsuspected intermediate priming step during isomerization. Some CMS mutations hinder isomerization of the liganded receptor, and some likely interfere with the priming step rather than with the channel gating step.

In Chapter 10, Srikanth Muppidi and Steve Vernino review the clinical spectrum of autoimmune and inherited peripheral nerve hyperexcitability (PNH) syndromes most of which are related to voltage gated potassium channels (VGKC). Knowledge of these syndromes advanced since 1999 due to the increased availability of anti-VGKC antibodies for evaluating autoimmune neuromyotonia, the identification of anti-VGKC antibodies in limbic encephalitis, and the discovery of additional mutations in VGKCs in hereditary ataxia-myokymia. A further interesting development has been that Caspr2 (contactin associated protein-like 2) and LGI1 (leucine-rich, gliomas inactivated 1) act as autoantigens in PNH and limbic encephalitis. The heterogeneity of antibodies and antibody specificities explains the diversity of clinical syndromes.

In Chapter 11, Jaap Plomp and Hugh Willison note that motor nerve endings at neuromuscular junctions express high levels of gangliosides and review recent experimental evidence for neuromuscular junction damage by anti-ganglioside antibodies, namely, GQ1b in the Miller-Fischer syndrome and anti-GM1 and anti-GD1a antibodies in the acute motor axonal neuropathy variant of the Guillain-Barré syndrome. However, the extent to which neuromuscular transmission is altered in humans by high titers of the antiganglioside antibodies is not yet defined.

I thank the contributing authors for their hard work that made this book possible and am grateful to Sid Gilman for encouraging me to edit the second edition. I am also indebted to the editorial staff of Oxford University Press, and especially Craig Panner and Kathryn Winder, for their help in bringing this book to fruition.

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MYASTHENIA GRAVIS AND MYASTHENIC DISORDERS

Second Edition

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Chapter 1

The Anatomy and Molecular Architecture of the Neuromuscular Junction

Andrew G. Engel

BASIC CONCEPTS

The neuromuscular junction (NMJ) is an anatomically and functionally differentiated chemical synapse, which transmits signals from the motor nerve terminal to the postsynaptic region of the muscle fiber. The position of NMJs on the muscle fiber, the configuration

of nerve terminals within the NMJ, and the differentiation of the postsynaptic region and complexity of the postsynaptic region within the NMJ vary according to phylum and species, between different muscles in a given species, and between different fibers in a given muscle. Despite these differences, all NMJs have five principal components: (1) a Schwann cell process, which caps the surface of the nerve terminal that faces away from the synaptic space; (2) a nerve terminal that contains the neurotransmitter; (3) a synaptic space, lined with basement membrane; (4) a postsynaptic membrane, which contains the receptor for the neurotransmitter; and (5) junctional sarcoplasm, which provides structural and metabolic support for the postsynaptic region (Figure 1–1). In vertebrate voluntary muscle, the neurotransmitter is typically acetylcholine (ACh), the receptor is a nicotinic ACh receptor (AChR), and the synaptic space contains endplate (EP)-specific species of ACh esterase (AChE). The presynaptic region consists of the nerve terminal covered by Schwann cell. The postsynaptic region consists of junctional folds lined by the postsynaptic membrane and the underlying junctional sarcoplasm.

The synaptic vesicles in the nerve terminal contain quantal packets of ACh. The vesicles release these spontaneously into the synaptic space, generating miniature EP potentials (MEPPs). When a nerve impulse depolarizes the presynaptic membrane, the ingress of Ca^{2+} into the nerve terminal through voltage-gated Ca^{2+} channels causes the release of a larger number of quanta. These generate a larger depolarization, the EP potential (EPP). Upon release of ACh into the synaptic space, AChE

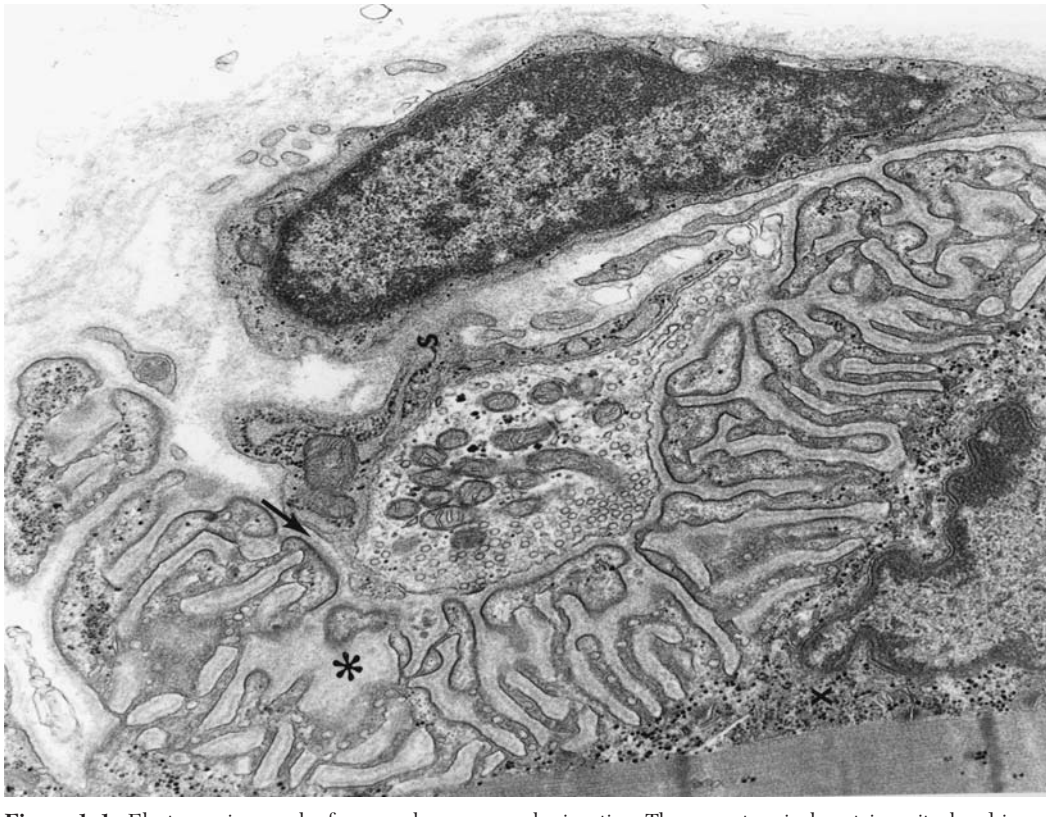

Figure 1–1. Electron micrograph of a normal neuromuscular junction. The nerve terminal contains mitochondria, small clear synaptic vesicles, giant synaptic vesicles (G), dense-core vesicles (arrowheads), agglutinated vesicles surrounded by membrane (asterisk), and active zones (arrows) in register with the secondary synaptic clefts. The terminal expansions of the junctional folds are covered by dense postsynaptic specializations. Numerous microtubules, other tubular and vesicular structures, ribosomes, and pinocytotic vesicles can be observed in the junctional folds. The junctional sarcoplasm contains abundant mitochondria, rough and smooth-surfaced endoplasmic reticulum, microtubules, and abundant microfilaments. S = Schwann cell process. x 40,400. (Reproduced from Engel[332] by permission.)

hydrolyzes some ACh molecules before they can reach the postsynaptic membrane. The remaining ACh molecules bind to AChRs and open AChR ion channels for exponentially distributed periods. When ACh molecules dissociate from AChR, they are rapidly hydrolyzed by AChE. Choline released by ACh hydrolysis is taken up by the nerve terminal via a sodium- and energy-dependent transport process. ACh is then resynthesized in the cytosol of the nerve terminal, from choline and acetate, in a reaction catalyzed by choline acetyltransferase. It is then packaged into synaptic vesicles by a specific transport mechanism.

All myasthenic disorders result from structural or functional alterations in one or more components of the NMJ. For example, a decreased number of presynaptic voltage-gated Ca^{2+} channels causes the Lambert-Eaton myasthenic syndrome (see Chapter 7). A decreased number of postsynaptic AChRs, together with destruction of the junctional folds, are cardinal features of autoimmune myasthenia gravis (see Chapter 3). Congenital or immune-mediated loss of function of the presynaptic voltage-gated K^+ channels produces excessive quantal release from the nerve terminal, and the clinical condition of neuromyotonia (see Chapter 10). Distinct congenital myasthenic syndromes result from specific defects in presynaptic, synaptic basal lamina-associated, and postsynaptic proteins (see Chapter 8). To facilitate understanding the mechanisms known to cause myasthenic disorders, this chapter reviews the classical anatomic features and current notions of the molecular architecture of the NMJ. The clinical, electrophysiological, and pathological aspects of the individual myasthenic disorders are discussed in subsequent chapters.

THE INNERVATION OF MUSCLE

Mature muscle receives a topographic projection of nerve fibers from its motor neuron pool.[1] The rostrocaudal axis of the motor pool is systematically mapped onto the rostrocaudal axis of the muscle. This is associated with segmental ordering of axons in the nerve and may be aided by axonal guidance at branch points in the nerve and by positional labels within the muscle.[2]

In mammalian muscles, most fibers are focally innervated near the center of the fiber by single NMJs (Figure 1–2A), and these fibers propagate action potentials. Intrafusal fibers, and a small proportion of extrafusal fibers in the extraocular,[3,4] facial, laryngeal, and lingual[3,5] muscles receive a distributed innervation consisting from two to many NMJs positioned on their surface at regular intervals. The EPPs on these fibers are propagated electrotonically, without triggering an all-or-none action potential. Cross-reinnervation studies during development indicate that the source of innervation determines the pattern of synaptic sites on the muscle fibers.[1,6]

Motor nerve endings are *plate-like*, *grape-like*, or *trail-like*. *Plate-like* nerve endings form round or elliptical loops on the muscle fiber surface (Figure 1–2B and C). On mature muscle fibers, the diameter of the innervated zone ranges from 10 to 80 μm with an average of 33 μm, and is proportionate to the diameter of the muscle fiber.[3] Plate-like nerve endings generate a propagated action potential. They are the most common moiety in mammals and reptiles, but also occur in birds and lower vertebrates.[7] *Grape-like* endings consist of a spray of fine varicose filaments that end in minute expansions.[8,9] They occur on fibers with distributed innervation that cannot propagate an action potential. However, not all muscle fibers with distributed innervation have grape-like terminals, and grape-like endings can also occur on focally innervated fibers.[1,3,10] *Trail-like* endings consist of fine branches bearing varicose dilations. They occur on multiply innervated bag_2-type and chain-type intrafusal muscle fibers supplied by γ-motor nerve fibers, and are concentrated in the juxtaequatorial region of the muscle spindle.[3]

THE PRESYNAPTIC REGION

The preterminal myelinated nerve fiber is surrounded by a sheath of perineural epithelial cells (Henle's sheath) that is partially surrounded by fibroblasts and other connective tissue elements (Figure 1–3). A basement membrane covers the myelinated nerve fibers and Henle's sheath, but not the fibroblasts. Within a few micrometers of the NMJ, the myelin sheath ends abruptly at the last node of Ranvier (Figures 1–2B and 1–3). Between the last node of Ranvier and the NMJ, the terminal axon is enveloped by the Schwann cell, which is surrounded by Henle's sheath (Figure 1–3).

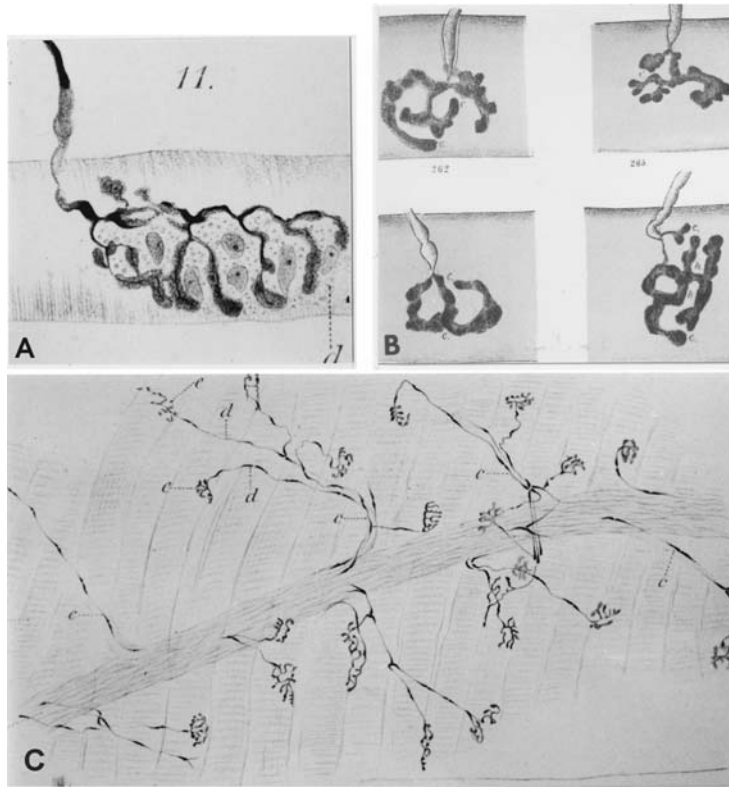

Figure 1–2. Early but very accurate camera lucida drawings of intramuscular nerve endings. (A) Intramuscular nerve branches and terminals in salamander muscle visualized by supravital staining. An intramuscular nerve coursing from lower left to upper right gives off small branches (C) that divide into single nerve fibers (D) that form terminals (E) on individual muscle fibers. The muscle fibers course vertically. The fine horizontal lines on the fibers represent cross striations. (B) Plate-like nerve terminal on a lizard muscle fiber, supravital staining. (C) Plate-like nerve endings on guinea pig muscle fibers visualized by a gold impregnation technique. (Panels A and B are reproduced from Dogiel, Arch. Mikroscop. Anat. 35:305–320, 1890; panel C is reproduced from Kühne, Z. Biol 23:1–148, 1887.)

Henle's sheath ends abruptly a short distance above the NMJ,[11] but the Schwann cell extends to cover that aspect of the nerve terminal that does not face the synaptic cleft (Figures 1–1 and 1–3). The basement membrane overlying the Schwann cell is continuous with the nonsynaptic basement membrane of the muscle fiber flanking the NMJ and also with the basement membrane lining the synaptic space. Therefore, only basement membrane separates the synaptic space from the extracellular space.

The Schwann cell process overlying the terminal axon and nerve terminal contains numerous microfilaments, smooth and rough endoplasmic reticulum, mitochondria, and, depending on the plane of the section, the cell's nucleus.

The terminal axon contains neurofilaments, microtubules, smooth endoplasmic reticulum, a variable number of mitochondria, and a few synaptic vesicles (Figure 1–3).

THE NERVE TERMINAL

The nerve terminal contains abundant, small, clear synaptic vesicles, fewer giant synaptic vesicles, dense-core vesicles, coated vesicles, mitochondria, and varying amounts of neurofilaments, microtubules, smooth endoplasmic reticulum, glycogen granules, lysosomal structures, and larger canaliculi and cisternae (Figures 1–1, 1–3, and 1–4). The relative abundance of the subcellular components in nerve terminals varies within a given NMJ with stage of development, aging, and neural activity. In the resting human NMJ, mitochondria occupy

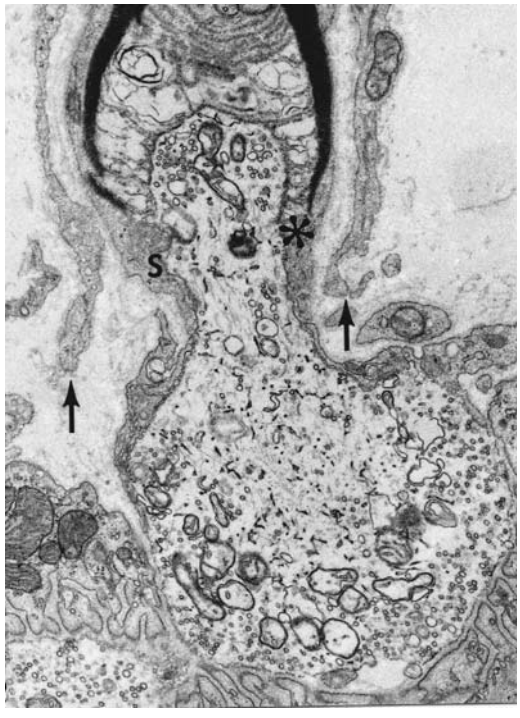

Figure 1–3. Approach of a preterminal nerve fiber to the NMJ. The myelin sheath ends abruptly at the last node of Ranvier (asterisk). The rest of the preterminal nerve fiber (p) is surrounded by Schwann cell processes (S). Henle's sheath terminates shortly beyond the last node of Ranvier (arrows). The preterminal axon (p) contains mitochondria, neurofilaments, and sparse synaptic vesicles. The basal lamina covers Schwann cells and Henle's sheath, and extends into the synaptic space. x 11,900. (Reproduced from Engel[332] by permission.)

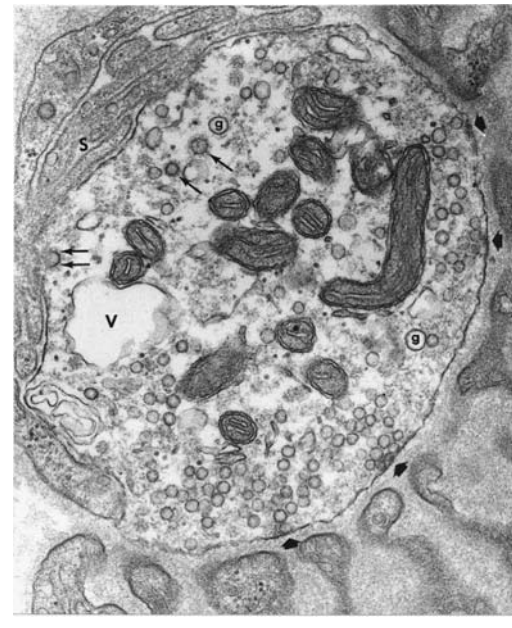

Figure 1–4. Nerve terminal in rat gastrocnemius muscle. Synaptic vesicles are concentrated near the presynaptic membrane; mitochondria cluster near the center of the terminal. A few giant vesicles (g) and coated vesicles (arrows) are present. A coated pit (double arrows) is budding from the axolemma covered Schwann cell (S). The nerve terminal also contains glycogen granules, a small vacuole (V), and an amorphous finely granular matrix. Solid arrows indicate four active zones that consist of dense spots on the inner surface of the presynaptic membrane and associated synaptic vesicles. The active zones are in register with the secondary synaptic clefts. Note dense membrane specializations on the terminal expansions of the junctional folds. x 63,000. (Reproduced from Engel[333] by permission.)

approximately 15% of the nerve terminal volume. There are 50 to 70 synaptic vesicles per μm^2 of the nerve terminal area.

Giant Synaptic Vesicles

Giant synaptic vesicles are two to three times larger than the small clear synaptic vesicles (Figures 1–4 and 1–5). They are relatively sparse, but increase in number after intense exocytotic activity.[12,13] They may arise from coalescence of smaller vesicles,[14] or may represent intermediates of membrane recycling.[12] Their appearance after prolonged transmitter release correlates with appearance of giant MEPPs, suggesting that they contain multiple ACh quanta.[12] Similar vesicles also appear in frog nerve terminals treated with vinblastine.[14]

Coated Vesicles

Coated vesicles are covered by clathrin.[15] In quick-freeze, deep-etch, rotary-shadow preparations, the coat consists of a polyhedral surface lattice.[16] They are relatively sparse, but increase in number after activity. They arise from endocytotic pits in the axolemma and pinch off from where the axolemma is covered by Schwann cell processes,[17] and they represent an intermediate in the synaptic vesicle cycle (Figure 1–5) (also see section below on Endocytosis).

Dense-Core Vesicles

Dense-core vesicles are 1.5- to 2-fold larger than the small clear vesicles, and are distributed randomly in the nerve terminal (Figure 1–5). These

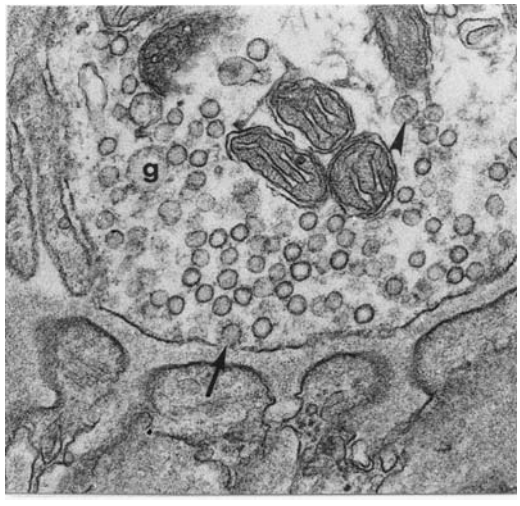

Figure 1–5. Part of a nerve terminal and underlying postsynaptic region. Note coated vesicle budding from the presynaptic membrane (arrow) and giant synaptic vesicle (g). The synaptic space is filled with basal lamina. x 51,800. (Reproduced from Engel[333] by permission.)

vesicles are morphologically and biochemically related to the secretory granules of endocrine cells. They are sparse in the mature nerve terminal but are relatively abundant in nerve growth cones and sprouts, and in regenerating nerve terminals.[18] In different parts of the nervous system, the dense-core vesicles contain neuropeptides, condensed proteins, or small nonprotein molecules. The presence of dense-core vesicles in adrenergic nerve terminals has been correlated with catecholamine associated fluorescence.[18,19] At the NMJ, the dense-core vesicles contain agrin, calcitonin gene-related peptide (CGRP),[20,21] and probably other neuroactive substances that can modify synaptic structure and function. Their release from the nerve terminal is regulated by calcium and Munc18-1[22] and modulated by synaptotagmin,[23] but the release from the nerve terminal differs from that of the small synaptic vesicles in several respects: the release is not preferentially at the active zones[24]; the rate of release is not enhanced by α-latrotoxin[21,25]; and the release occurs relatively slowly, after about 50 ms of high-frequency stimulation that results in a gradual increase of calcium concentration in the depth of the nerve terminal.[25,26] Following exocytosis, the dense-core vesicles are recycled, but their refilling requires a passage through the Golgi system.[27]

Small Clear Synaptic Vesicles

The smooth-surfaced clear synaptic vesicles with a mean diameter of 50 to 60 nm represent the predominant vesicle species in the nerve terminal,[28,29] and hereafter will be referred to simply as *synaptic vesicles*. Their lumen contains ACh, ATP, GTP, a relatively high concentration of calcium and magnesium ions, and a vesicle-specific proteoglycan.[30–33] The vesicles are more abundant near the presynaptic membrane than elsewhere in the terminal, whereas mitochondria and other organelles are concentrated in the center and upper part of the terminal.[34–36] They tend to be focused over dense spots on the presynaptic membrane that are part of the active zones, where synaptic vesicles exocytose their contents into the synaptic space[35,37–40] (Figures 1–4 and 1–6).

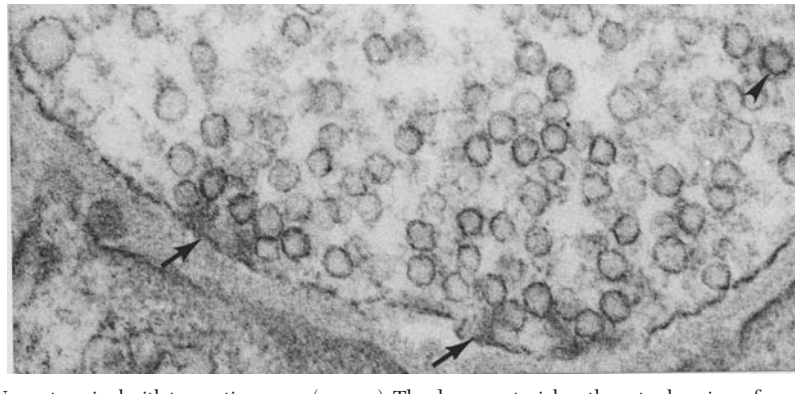

Figure 1–6. Nerve terminal with two active zones (arrows). The dense material on the cytoplasmic surface of the active zone surrounds the associated synaptic vesicles. The synaptic space contains strands of basal lamina. The crests of two junctional folds face the active zone. Arrowhead points to a coated vesicle. x 114,000. (Reproduced from Engel[333] by permission.)

Synaptic vesicle precursors, associated with different sets of synaptic vesicle proteins, are produced in the body of the anterior horn cell and then are carried to nerve terminals by kinesin-like motors via fast axonal transport[41–44] by means of tubulovesicular organelles.[45] Further maturation of the vesicle precursors and their packaging with ACh occurs within the nerve terminal. A reduced number of synaptic vesicles, associated with a decrease in the number of readily releasable quanta, occurs in a congenital myasthenic syndrome. The putative cause of the syndrome is impaired axonal transport of synaptic vesicle components to the nerve terminal (see Chapter 8).

A current atomic model of the synaptic vesicle indicates that ~20% of its membrane is occupied by more than 400 different proteins. Because transmembrane regions of proteins are surrounded by a ring of fixed phospholipids, the vesicle membrane is likely rigid.[46,47] Some vesicular proteins are more abundant than others: there are 70 copies of synaptobrevin, 30 of synaptophysin-1, and 15 of synaptotagmin-1 per vesicle, but there is only one copy of the vesicular proton pump, though this large molecule accounts for more than 10% of the vesicle protein.[46,47] Functions of these proteins will be discussed in subsequent sections of this chapter.

The functions and activities of the synaptic vesicles include: (1) the concentrative uptake and storage of ACh; (2) movement to and docking at the active zones; (3) fusion with the presynaptic membrane to release ACh by exocytosis; and (4) recycling.[27,48–50] During recycling, the vesicles are retrieved from the presynaptic membrane, and then are recharged with ACh. Performance of these tasks requires the interaction of highly specialized vesicular, cytosolic, and target membrane proteins.

Vesicular ACh Uptake

The synthesis of ACh from choline and acetate takes place in the cytoplasm of the nerve terminal in a reaction catalyzed by choline acetyltransferase (ChAT). Uptake of the newly formed ACh into the synaptic vesicles is mediated by a vacuolar proton-pump ATPase that lowers the intravesicular pH and drives ACh uptake through the vesicular ACh transporter (VAChT).[51–53] The same VAChT transiently exports ACh from the nerve terminal during exocytosis when the inner surface of the vesicular membrane is exposed to the synaptic space. Decreased resynthesis of ACh causes a highly disabling congenital myasthenic syndrome associated with abrupt episodes of apnea[54] (see Chapter 8).

The entire coding region of the *VACHT* gene is contained in the first intron of the *CHAT* gene, and the two genes share common regulatory elements for transcription.[55] The structural information that specifically targets VAChT to the synaptic vesicles resides within the cytoplasmic C-terminal domain of VAChT. An isoform of VAChT, VMAT2, is targeted to the large, dense-core synaptic vesicles, where it subserves the concentrative uptake of neuroactive substances other than ACh.[56]

Synaptic Vesicle Pools

Three functionally distinct synaptic vesicle pools have been identified in different synaptic systems by electron microscopy studies, by fluorescence microscopy of the position and movement of synaptic vesicles labeled with styryl dyes, by electrophysiology recordings of capacitance changes when synaptic vesicles fuse with the presynaptic membrane, and by monitoring the postsynaptic voltage response to released transmitter quanta.[57]

A readily releasable pool comprises synaptic vesicles primed for release and in physical contact with the presynaptic membrane where it comprises the active zone. This pool accounts for ~1–2% of all synaptic vesicles in the nerve terminal. However, not all vesicles above the active zones are primed for release. Vesicles from this pool exocytose at the onset of physiologic stimulation, are recycled within a few seconds, and then rapidly mix with a recycling pool.

The recycling pool accounts for ~10–20% of all synaptic vesicles in the nerve terminal. Vesicles from this pool exocytose within a few seconds after the start of stimulation, are recycled within a few seconds, and then rapidly replete the readily releasable pool and slowly mingle with a reserve pool of vesicles.

The reserve pool comprises ~80–90% of all synaptic vesicles in the nerve terminal. Vesicles from this pool exocytose only after stimulation lasting tens of seconds or minutes, are recycled

slowly over minutes, and mix slowly with other vesicle pools. Although the three vesicle pools are functionally distinct, only the readily releasable vesicles have a distinct anatomic locus at the active zones; vesicles in the recycling and reserve pools are intermingled above the active zones and in deeper parts of the nerve terminal and cannot be identified by simple inspection of electron micrographs.[57]

Synaptic Vesicles Move to and Dock at the Active Zones

Vesicles in the recycling pool are thought to move to the active zones by simple diffusion.[58] Movement of the synaptic vesicles from the reserve pool involves several proteins.

Synapsin I, a synaptic vesicle-specific phosphoprotein, links synaptic vesicles to the cytoskeleton.[59,60] The hydrophobic carboxy terminus of synapsin I is attached to the synaptic vesicles by binding to Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II).[61] Other domains of synapsin I bind to cytoskeletal actin, spectrin, and tubulin and thus anchor the vesicles to the cytoskeleton. In addition, synapsin I promotes the polymerization of actin monomers into actin filaments and the formation of thick bundles of actin filaments.

During physiological activity, Ca^{2+}-dependent phosphorylation of synapsin I by CaM kinase II decreases the affinity of synapsin I for CaM kinase II, releasing the vesicles from cytoskeletal constraints.[59,61] The synaptic vesicles in the reserve pool can move closer to the active zone. Filamentous actin and myosin likely play a role in this movement because: (1) blocking actomyosin dependent movements by inhibiting myosin light-chain kinase restricts vesicle release in hippocampal synapses[62]; (2) staurosporine, an inhibitor of a wide spectrum of protein kinases, impairs vesicle movement from the reserve pool to the active zones[63]; and (3) disruption of actin restricts movement of vesicles from the reserve pool at the *Drosophila* NMJ[64–66]; and (4) C-terminal residues of NCAM play a role in activating myosin light-chain kinase and hence myosin II, and NCAM-deficient mice show a cyclic failure of neuromuscular transmission on high-frequency stimulation.[67–69] These observation support the notion that that actin filaments serve as tracks for movements of the vesicles by myosin motors.[64]

Following mobilization, the synaptic vesicles must be docked at the active zones for their efficient exocytotic release by Ca^{2+}. It was previously thought that docking was due to the formation of a complex between synaptobrevin on the synaptic vesicles, and syntaxin and SNAP-25 on the presynaptic membrane. However, neither cleavage of these three proteins by clostridial neurotoxins nor deletion of syntaxin in *Drosophila* prevents vesicle docking.[70] Recent studies suggest that the interaction of vesicular synaptotagmin with neurexin and the voltage-gated Ca^{2+} channel on the presynaptic membrane plays a role in vesicle docking (also see below, under The Active Zone and the Voltage-Gated Ca^{2+} Channels).

Exocytotic Machinery

Ca^{2+}-regulated exocytosis of the synaptic vesicles involves the coordinated interaction of highly conserved proteins located on the synaptic vesicle, in the cytosol, and on the presynaptic membrane. The key proteins involved include: (1) synaptobrevin and synaptotagmin, associated with the synaptic vesicles; (2) NSF (N-ethylmaleimide sensitive ATPase) and α-SNAP (soluble NSF attachment protein), in the cytosol; and (3) syntaxin, SNAP-25 (synaptic vesicle associated protein of 25 kDa), and voltage-gated Ca^{2+} channels associated with the presynaptic membrane. Because isoforms of several of these and of other exocytosis-related proteins play a universal role in vesicle-target membrane fusion in eukaryotic cells, the specificity of the docking and fusion process at the different vesicle-target membrane sites in eukaryotic cells must depend on the specificity of receptors on the vesicular and target membranes and presence of other accessory molecules.[71,72] In addition to these key proteins, numerous other proteins have been implicated in synaptic vesicle exocytosis in model systems that include hippocampal neurons in culture, *Drosophila*, *C. elegans*, and the frog NMJ.

SNARE Complex

Vesicular synaptobrevin, together with presynaptic membrane syntaxin and SNAP-25, serve

as a receptor for α-SNAP. Therefore, synaptobrevin is referred to as a v-SNARE (vesicular SNAP receptor), and syntaxin and SNAP-25 are called t-SNAREs (target membrane SNAP receptors).[70] A key role of t- and v-SNAREs in exocytosis is evidenced by evaluating the effects of clostridial neurotoxins on their action. Botulinum toxins B, D, F, and G, and tetanus toxin cleave synaptobrevin; botulinum toxins A and E cleave SNAP-25; and botulinum toxin C cleaves syntaxin.[73,74] In each case, the result is an arrest of exocytosis.

Biophysical,[75–78] quick-freeze/deep-etch electron microscopy,[79] and crystallographic[80] studies have elucidated the structure and function of the SNARE complexes.[72,81,82] The cytoplasmic portion of each SNARE protein contains repeats of 7 amino acids that can assume an α-helical conformation. Monomeric SNAREs are largely unstructured; after combining with each other, they become highly α-helical, assume a coiled-coil configuration, and acquire enhanced thermodynamic stability.

Assembly of the t- and v-SNARES occurs in three steps. First, monomeric SNAP-25, anchored to the presynaptic membrane by palmitoyl side chains, binds two molecules of syntaxin, and the complex assumes a coiled-coil configuration. Second, the v-SNARE synaptobrevin binds to the preassembled t-SNARES by displacing one of the two syntaxin molecules bound to SNAP-25. The entire complex is now a coiled-coil in which α-helices are strongly held together by hydrophobic interactions. The complex, also referred to as the SNARE-pin, is a 12- to 14-nm-long and ~2-nm-wide cylindrical bundle, with the N-termini of each component at one end and the C-termini at the membrane anchor end. Third, the complex is stabilized by complexin, a small soluble neuronal protein, that binds to the complex in an antiparallel α-helical conformation to seal the groove between synaptobrevin and syntaxin[83] (see Figure 1–7). Complexin has now been shown to act as a reversible clamping protein that can freeze the SNARE-pin as an assembled intermediate en route to fusion. When calcium binds to the calcium sensor synaptotagmin, the complexin clamp is released[84] (also see section below on Synaptotagmin-1). Because the v-SNAREs and t-SNAREs are anchored in two different membranes facing each other, the formation of SNARE-pins brings vesicle and target membranes into very close proximity. This, together with a strong basic charge at the C-terminal end of the SNARE-pins, may provide the driving force behind membrane fusion.[80] Multiple SNARE-pins are probably needed to trigger fusion of a single synaptic vesicle, and the pins likely are arranged in a ring-like structure at the contact point.[85] When v- and t-SNAREs are reconstituted into separate liposomal vesicles, they assemble to form SNARE-pins that link adjacent vesicles[85]; this process is inefficient but is dramatically

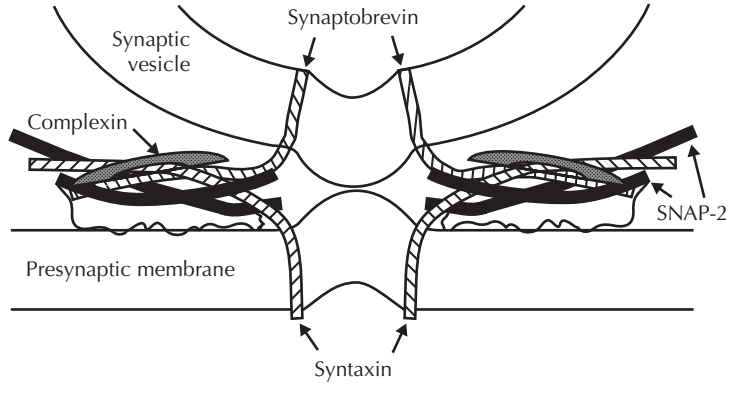

Figure 1–7. A model of the SNARE complex formed between the v-SNARE synaptobrevin and the t-SNAREs syntaxin and SNAP-25. Two complexes are imaged. Synaptobrevin and syntaxin are anchored by transmembrane regions in the lipid bilayer of the synaptic vesicle (above) and presynaptic membrane (below), respectively. SNAP-25 is linked to the presynaptic membrane by a polypeptide chain (indicated by a thin undulating line). The cytoplasmic domains of the v- and t-SNARES form a coiled-coil that pulls the synaptic vesicle and the presynaptic membrane into close proximity. Impending fusion is suggested by bulging regions of the vesicular and presynaptic membranes. This diagram is based on models proposed by Sutton et al.[80] and by Weber et al.[85]

accelerated when a stabilized syntaxin/SNAP-25 acceptor complex is used.[86]

Priming of Docked Synaptic Vesicles

A proportion of the synaptic vesicles in the nerve terminal are recruited and docked on the plasma membrane of the nerve terminal. However, only a fraction of these vesicles is in a primed state capable of calcium-evoked fusion, that is, in a readily releasable state. Munc13 (Unc13 in *C. elegans*) is required for vesicle priming. Munc13 stabilizes the open conformation of syntaxin that was forced into a closed conformation by Munc18; this drives syntaxin to interact with SNAP-25 and synaptobrevin and thus to form a SNARE-pin. Tomosyn, on the other hand, inhibits this interaction.[87] Thus, priming is modulated by the balance between tomosyn and Munc13 that likely regulates the availability of open-syntaxin.[88–90]

Synaptotagmin-1

Synaptotagmin-1, a 65 kDa molecule, belongs to a large family of membrane proteins involved in membrane fusion in brain and other organs.[91] Synaptotagmin-1 has a short, glycosylated intravesicular N-terminal domain, a transmembrane domain, and a cytoplasmic domain that harbors two Ca^{2+} regulatory C2 domains (C2A and C2B) connected by a short linker and separated from the transmembrane domain by a highly charged sequence.

The C2A domain binds phospholipids and two Ca^{2+} ions held in position by five negatively charged aspartate residues on two peptide loops.[92] Fast vesicle exocytosis is driven by Ca^{2+}-dependent biding of synaptotagmin to the SNARE complex and to membrane phospholipids.[93]

The Ca^{2+} concentration for half-maximal binding (EC50) is 200 μM, attained only in close proximity to the active zones. When the C2A domain binds Ca^{2+}, it acquires a large positive electrostatic potential and then binds syntaxin.[94] Both syntaxin and synaptotagmin are highly associated with the presynaptic voltage-gated Ca^{2+} channels.[95]

The C2B domain of synaptotagmin binds to β-SNAP, which like α-SNAP binds to NSF, polyinositol phosphates, and the vesicular protein SV2A.[91,96] Ca^{2+} inhibits the interaction between SV2A and synaptotagmin, with an EC50 of 10 μM. The C-terminus of synaptotagmin binds neurexin, the presynaptic membrane receptor for α-latrotoxin (the active component of black-widow spider venom that causes massive exocytosis). The C2B domain also participates in endocytosis (see below under Endocytotic Events and the Formation of New Synaptic Vesicles). Finally, an unknown region of synaptotagmin binds Munc13.[91] In mutant mice deficient in synaptotagmin, the Ca^{2+}-dependent evoked synaptic response is severely depressed. Consequently, the animals die shortly after birth.[97]

Synaptotagmin also binds to the voltage-gated Ca^{2+} channel as well as to syntaxin and neurexin, which, in turn, are attached to the presynaptic Ca^{2+} channel.[95,98] Hence, synaptotagmin also could participate in docking. Activation of synaptotagmin by Ca^{2+} is a likely trigger for exocytosis.[91,99] Recent studies have shed further light on the manner in which synaptotagmin regulates synaptic vesicle exocytosis by interaction with complexin. In a model system, SNARE proteins were flipped, so that instead of being expressed on intracellular membranes, they were exposed on the cell surface. Cells expressing such flipped SNAREs fused spontaneously. The introduction of complexin clamped the SNARE-pins after they began to assemble but before they `were fully zippered, and thereby prevented cell-cell fusion. Adding synaptotagmin and then calcium to the SNARE-complexin intermediate again allowed cell-cell fusion. According to this attractive model, synaptotagmin couples the calcium signal to SNAREs in a mechanism that requires complexin.[84]

Finally, in addition to its function as a calcium sensor, synaptotagmin also plays a role positioning synaptic vesicles for synchronous release.[100]

Steps in Exocytosis

On the basis of recent studies, vesicle exocytosis can be postulated to involve the following major steps:

1. Partial and reversible assembly of the SNARE complex primes docked synaptic

vesicles for exocytosis prior to arrival of the Ca^{2+} trigger.[101]
2. Opening of the voltage-gated Ca^{2+} channels (VGCCs) transiently increases the intracellular Ca^{2+} concentration from <1 μM to >10 μM near the VGCCs and promotes Ca^{2+} binding to synaptotagmin.[102] This enables synaptotagmin to interact with syntaxin and SNAP-25,[103–105] and to inhibit the clamping effect of complexin on SNARE-pin assembly.[84]
3. Syntaxin and SNAP-25 now firmly engage synaptobrevin to complete formation of the SNARE-pins.
4. Full assembly of the SNARE-pins brings vesicular and target membranes into close proximity, which initiates membrane fusion in a probabilistic manner.[106]

Other Proteins Modulating Exocytosis

RAB3A, RABPHILIN-3A, AND RIM

Rab3A, a small GTP-binding protein, is also implicated in synaptic vesicle docking and fusion.[107] The Rab proteins belong to the p21ras superfamily, whose members regulate membrane fusion-fission events by cycling between membrane-bound and membrane-free states. When attached to a synaptic vesicle, Rab3A binds GTP. Rab3A-GTP binds Rabphilin-3A, a cytosolic protein with zinc-finger and C2 domains, and RIM.[108] Both Rabphilin-3A and RIM bind to Rab3A through sequences contained in their zinc-finger domains.

At the time of exocytosis, activation of a GTPase converts Rab3A-GTP to Rab3A-GDP, whereupon both Rab3A-GDP and Rabphilin-3A dissociate from the synaptic vesicle.[108–110] Subsequently, Rab3A-GDP becomes attached to another synaptic vesicle and recaptures GTP by nucleotide exchange. Evidence to date suggests that Rab3A decreases the probability of quantal release,[111] RIM promotes transmitter release,[108] and Rabphilin-3A plays a regulatory role in both exocytosis and endocytosis.[112] The binding of Rabphilin to SNAP-25 regulates exocytosis after the readily releasable pool of synaptic vesicles has been exhausted.[113]

A recent study shows that RIM determines the specific localization of the presynaptic P/Q type Ca^{2+} channels at the active zones via direct Ca^{2+} channel/PDZ-domain interaction and via indirect binding of the Ca^{2+} channels to a RIM-binding protein (RIM-BP). In addition, RIMs form an N-terminal priming complex with Rab3 and Munc13 in which Munc13 acts by binding to the SNARE complex.[114,115]

RAB5

This protein is involved in endosomal fusion events in different tissues and is present on the synaptic vesicles in high concentrations. It likely plays a part in interaction between the vesicles and endosomes during the vesicle cycle. Mutations in Rab5 in *Drosophila* impair evoked transmitter release.[116]

SV2

This protein is found in synaptic vesicles and endocrine cells. It is present in two major (SV2A and SV2B) and one minor (SV2C) isoforms. As noted above, SV2A interacts with the C2b domain of synaptotagmin in the absence of Ca^{2+}. The major phosphorylation site of SV2 is at its cytoplasmic amino terminus, and phosphorylation increases its affinity for synaptotagmin.[117] Neurons lacking both SV2 isoforms show increased Ca^{2+} dependent transmitter release[118] and increased resting and evoked Ca^{2+} levels.[119]

SYNAPTOPHYSIN

Synaptophysin, a 38 kDa glycoprotein, is an abundant integral membrane protein of the synaptic vesicles.[120] It has properties of a cation selective channel, with higher selectivity for K^+ than other cations, but is impermeable to Ca^{2+}.[121] It is phosphorylated by a tyrosine kinase,[122] interacts with a subunit of the vacuolar proton pump,[123] and may interact with synaptobrevin during exocytosis.[124] When overexpressed at the *Xenopus* NMJ, synaptophysin increases the frequency of spontaneous quantal release and augments the number of quanta released by nerve impulse.[125] In the yeast two-hybrid system, synaptophysin interacts with the AP1-adaptor protein, γ-adaptin, and may thus play a role in endocytosis. However, synaptophysin null mice show no functional or morphologic abnormality.[126]

CYSTEINE STRING PROTEIN (CSP), HEAT SHOCK PROTEIN 70 (HSC70), AND SGT CHAPERONE COMPLEX

These three proteins interact with each other to form a stable trimeric complex located on the surface of the synaptic vesicles. The complex functions as an ATP-dependent chaperone reactivating denatured substrates.[127]

CSP itself is a 34 kDa protein anchored via palmitoyl groups to the synaptic vesicle so that its C- and N-termini are cytoplasmic.[128,129] CSP harbors an N-terminal J domain, characteristic of heat-shock proteins, and a central multiply palmitoylated string of cysteine residues. By increasing the ATPase activity of Hsc70, CSP co-chaperones with Hsc70 to promote the formation or dissociation of protein complexes and to regulate conformational changes in proteins.[130,131] CSP also binds to the P/Q type Ca^{2+} channel with high affinity[132] and interacts with synaptotagmin.[133] In *Drosophila* mutants lacking CSP, the exocytotic machinery is preserved but calcium entry into the nerve terminal, calcium activation of exocytosis, or both, are impaired.[134] Injection of CSP into the chick ciliary neuron increases the Ca^{2+} current owing to recruitment of dormant Ca^{2+} channels.[135] In mice deficient in the CSPα isoform, the NMJ degenerates, synaptic transmission is impaired, and the mice die at ~2 months of age.[136]

Presynaptic Cytoskeletal Components

Quick-freeze, deep-etch electron microscopy shows that the main cytoskeletal elements in the nerve terminal consist of actin filaments and microtubules.[137–140] The actin filaments honeycomb the nerve terminal. They are most closely packed adjacent to the synaptic membrane, and become more sparse with distance from the membrane. Those filaments terminating against the active zone tend to be perpendicularly oriented to the presynaptic membrane. The filaments are straight, often intersect, and extend from vesicle to vesicle, and from vesicle to presynaptic membrane. The actin filaments are linked to the synaptic vesicles by approximately 30-nm-long filaments that represent single synapsin I molecules. Synapsin I molecules also link microtubules to the synaptic vesicles and crosslink the microtubules.[137] A similar cytoskeletal network exists in Purkinje cell dendrites.[140] Different cytoskeletal components have been found at the active zones. A recent tomographic cryo-electron microscopy study has revealed that the docked vesicles are interlinked by >5 nm long fine filaments; these give way that give way to multiple shorter filaments once the vesicles enter the readily releasable pool[140a]. The molecular identity of the fine tethering filaments has not been determined.

The Active Zone and the Voltage-Gated Ca^{2+} Channels

The active zone is an anatomically differentiated region that defines the site of synaptic vesicle docking and fusion. They are positioned in register with the secondary synaptic clefts that are flanked by the AChR-enriched crest of the junctional folds. Each active zone consists of a dense spot adjacent to the presynaptic membrane, synaptic vesicles focused on these spots (Figures 1–4 and 1–6), and voltage-gated Ca^{2+} channels in the adjacent presynaptic membrane. The dense spot contains a matrix of interconnected fibrils and particles that participate in vesicle exocytosis and membrane retrieval.[141] Proteins within the matrix of the active zone include RIM1, CAST, Bassoon, Munc13-1. CAST binds directly to Bassoon and to RIM1 (Rab interacting molecule 1) and indirectly to Munc13-1. RIM1 and Munc13-1 bind to each other. RIM1 binds to the C-terminus and Bassoon binds to the central region of CAST. RIM1 and Munc13-1 are implicated in synaptic vesicle priming. *RIM* null animals show a severe defect in spontaneous or evoked vesicle release but can dock vesicles at the active zone. Other proteins present in the active zones are considered below in the paragraphs on voltage-gated Ca^{2+} channels. Thus, the active zone matrix is a network of proteins important for regulating synaptic vesicle exocytosis and organization of the active zone.[142–144]

In the freeze-fractured presynaptic membrane, the Ca^{2+} channels appear as 10 to 12 nm integral membrane particles arrayed in double parallel rows (Figure 1–8). Ultra-rapid freezing of the NMJ within a few ms after stimulation, followed either by freeze-fracture or by