

## Miniseries: Illustrating the Machinery of Life

### Neuromuscular Synapse\*

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**Diverse biological data may be used to create illustrations of molecules in their cellular context. I describe the scientific results that support a recent textbook illustration of the neuromuscular synapse. The image magnifies a portion of the synapse at one million times, showing the location and the form of individual macromolecules. Results from biochemistry, electron microscopy, and X-ray crystallography were used to create the image.**

**Keywords:** cellular biology, molecular biology, molecular graphics and representations, molecular visualization.

A bewildering amount of biological data is available today in published journals and online. For the past few decades, I have worked to create a series of illustrations of the environment inside living cells, supported by this diverse cellular and biomolecular data. The goal of these illustrations is to present a significant portion of a living cell, showing all of its molecular components. I recently completed a new series of illustrations for the second edition of the book “The Machinery of Life” [1]. This report, and ones that will follow, will present the science behind these illustrations.

#### NEUROMUSCULAR SYNAPSES

The illustration of the neuromuscular synapse starts with an electron micrograph, which shows the overall ultrastructure (Fig. 1, taken from ref. [2]). The portion included in the illustration is 180 nm wide by 250 nm high, which is large enough to include several synaptic vesicles inside the axon of the neuron, the synaptic cleft with basement membrane, and part of the muscle cell cytoplasm. One of the distinctive junctional folds of the postsynaptic membrane is also shown, but due to the limited size of the illustration, the depth of the groove is not as pronounced as is often observed.

Whenever possible, the shape and size of each molecule is based on the atomic structures from the RCSB Protein Data Bank (<http://www.pdb.org>) [3], otherwise structures are taken from electron microscopy or inferred

from genetic sequences. I attempted to include molecules of the entire synaptic cycle in this illustration, as shown in Fig. 2 with a key in Fig. 3. At upper left, a synaptic vesicle is caught in the process of tethering to the axon membrane. The vesicle at upper right has fused with the membrane and is releasing its load of acetylcholine. Some of this acetylcholine is bound to receptors on the muscle surface at the bottom. The synaptic cleft contains several acetylcholinesterase molecules, which destroy the neurotransmitter. Then, a specific transporter, shown at the far right end of the axon membrane, transports the choline back into the axon. Finally, the enzyme choline acetyltransferase recreates the neurotransmitter.

Of course, many approximations must be made when creating such an image. Molecular structures are taken from the studies that are currently available, and often come from widely different organisms. The illustration is also designed to tell a biochemical story in an idealized synapse, so all of the molecules may not actually be expressed in the proportions shown here in a particular synapse.

#### SYNAPTIC VESICLES

The synaptic vesicles were based on a recent multidisciplinary study of purified vesicles [4]. This comprehensive study provided a detailed list of the composition and number of molecules, as well as providing a three-dimensional model of the entire vesicle. An additional review [5] provided detailed topological information for proteins embedded in the vesicle membrane. The hexameric ring of synaptophysin and its interaction with the SNARE assembly is taken from a 3D electron micrograph reconstruction [6]. I have speculatively drawn it as organizing the docking of the vesicle shown on the left, and then

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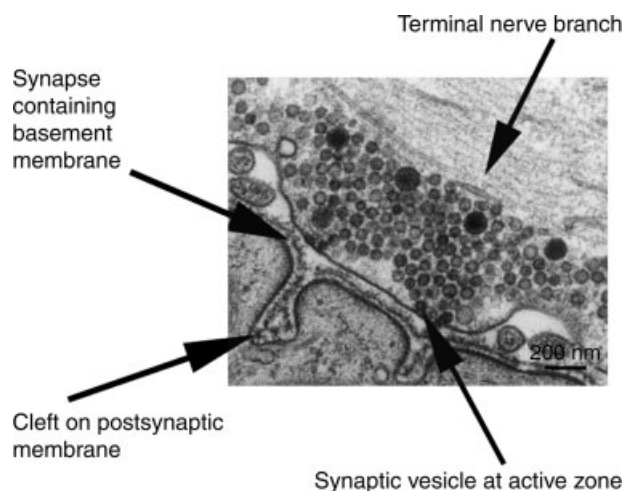


FIG. 1. **Electron micrograph of a neuromuscular synapse.** Reproduced with permission from Oxford University Press, N. P. Hirsch (2007) Neuromuscular junction in health and disease, *Br. J. Anesth.* 99(1), 132-138.

breaking up during the fusion event shown in the vesicle on the right. The interaction of synaptotagmin and the SNARE proteins synaptobrevin, syntaxin, and Snap25 [7] is taken from a crystal structure of intracellular portions of the complex [8]. The vacuolar ATPase was modeled after ATP synthase [9]. The transport protein that transports neurotransmitters into the vesicle is also included. It is taken from a homology model for VGLUT [10], the protein that transports glutamate into vesicles in excitatory neural cells.

Estimates of the amount of neurotransmitter vary widely with different sources. Takamori *et al.* [4] estimated about 1,790 molecules in their vesicle model (based on a concentration of 150 mM), and Hirsch [2] estimated 5,000–10,000 molecules of acetylcholine in a typical neuromuscular vesicle. Given an inner diameter of about 30 nm for the vesicle, this corresponds to a distance of 1.4–2 nm between neurotransmitter molecules.

Rab proteins, ras-family GTPases involved in vesicle trafficking, are also included on the surface of the vesicles. The structure was taken from PDB entry 3rab [11], and the UniProtKB entry P20336 indicates that there are two lipid anchors. Rab3A is shown at several different points in its cycle, as described in the review article by Sudhof [5]. In the undocked vesicles at the top, it is associated with rabphilin3A. In the docked vesicle, it is shown interacting with RIM in the presynaptic active zone [12]. After docking, it is removed from the axon membrane by GDI (rab GDP dissociation inhibitor) and recycled.

#### PRESYNAPTIC ACTIVE ZONE

Ultrastructural studies of synapses have revealed a protein-rich region inside the nerve terminal at the site of vesicle fusion and neurotransmitter release [13]. This active zone is thought to be a complex of hundreds of proteins embedded in the axon membrane and extending into the nerve cell cytoplasm, which together guide vesicles to the surface and mediate fusion and release.

Voltage-gated calcium channels are placed at the center of the active zone in many models and are intimately involved with neurotransmitter release [14, 15]. On the synaptic side, I have shown it interacting with the long arm of laminin [16]. Also included in this region is the receptor protein tyrosine kinase LAR (leukocyte common antigen-related receptor protein tyrosine kinase), which interacts with both intracellular molecules and molecules in the synaptic cleft [17]. The structure shown in the illustration is based on PDB entry 2v5y [18]. An integrin [19] is also included, based on the crystal structure of the extracellular segment in PDB entry 1jv2 [20].

A large scaffold of proteins is found inside the axon membrane, which presumably guides vesicles to the site of fusion. This scaffold is composed of many proteins, including small multidomain proteins and long structural proteins. I based the domain structure and connectivity of proteins in this region on schematic diagrams from several papers [21–24], including the proteins bassoon, CAST (CAZ-associated structural protein), Munc-13/18 (mammalian homolog of unc), RIM (Rab3-interacting protein), liprin, and MINT.

The abundant protein synapsin plays an important role in the clustering of synaptic vesicles in the active zone [25]. It is shown here as a dimer with shape based on the results from electron microscopy [26]. I have shown it forming a speculative linkage between actin filaments and the synaptic vesicles. Also shown in the neuron cytoplasm is NSF, the AAA+ protein that disassembles the SNARE complex (PDB entry 1d2n [27]), and choline acetyltransferase, the enzyme that forms acetylcholine (PDB entry 1t1u [28]).

To finish up the synaptic cycle, choline must be taken back into the neuron, to be recycled for the next signal. The sodium-coupled transporter CHT1 performs this task [29]. The structure shown here is based on the structure of the bacterial sodium-coupled leucine transporter LeuT from PDB entry 2qei [30].

#### SYNAPTIC CLEFT

The synaptic cleft in neuromuscular synapses contains a specialized synaptic basal lamina [31]. It includes some of the typical members of basement membranes, including collagen IV, laminins, perlecan, and entactin/nidogen. The perlecan structure was based on the information in a review [32]. The basement membrane also includes specialized components, including agrin and acetylcholinesterase. Agrin is a large proteoglycan that is important for the clustering of acetylcholine receptors on the postsynaptic membrane [33]. It is concentrated at the crests of the junctional folds, which is also the primary location of the receptors on the membrane [31]. On the basis of the results from electron microscopy [34], I have shown it as a snake-like protein with three major sites of glycosylation, binding to laminin at the N-terminal end and binding to dystroglycan at the C-terminal end.

In the neuromuscular synapse, acetylcholinesterase is found predominantly as a complex with the collagen-like



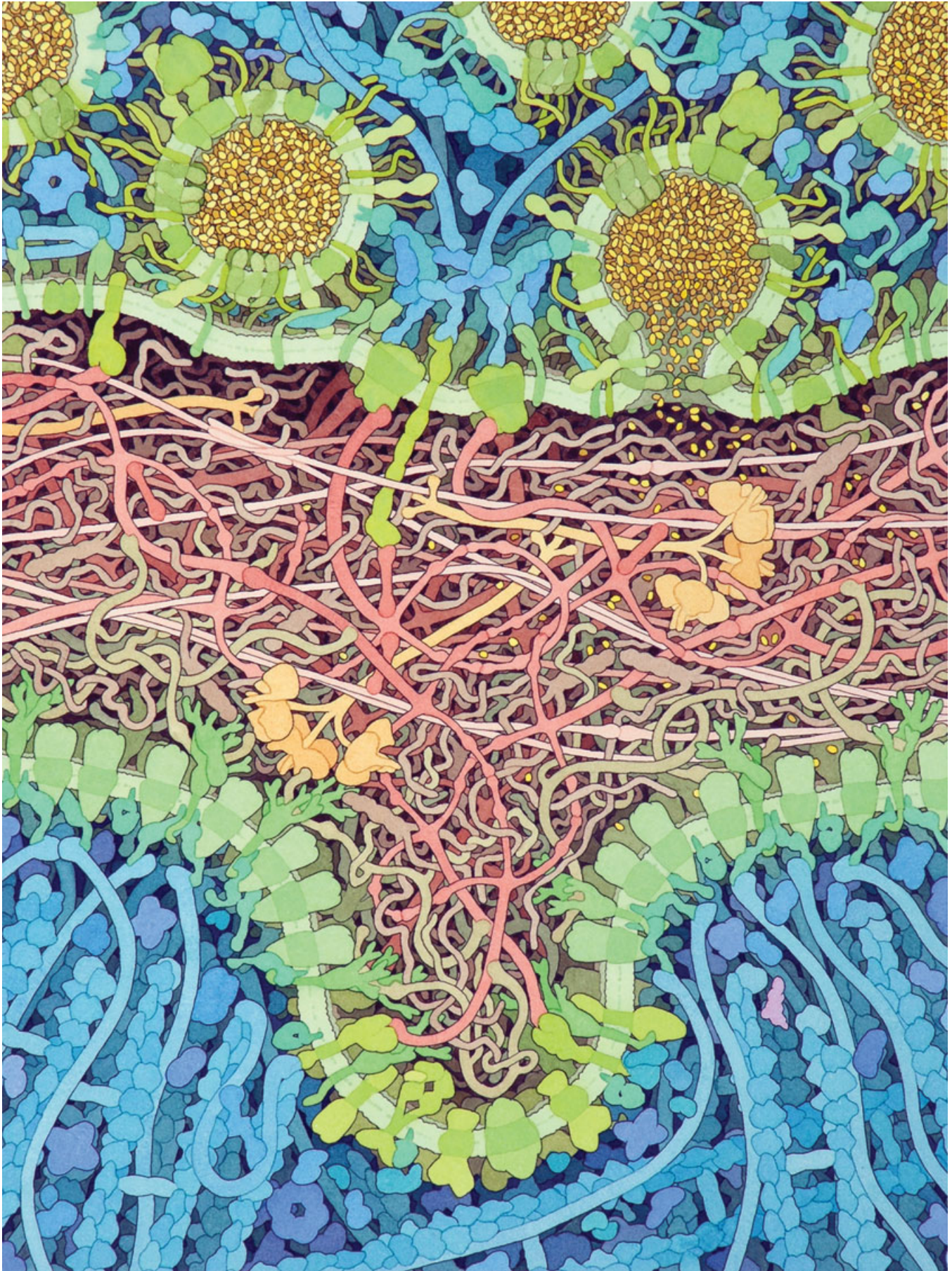


FIG. 2. **Illustration of a neuromuscular synapse, showing all macromolecules and acetylcholine at a magnification of  $\times 1,000,000$ .** At this magnification, individual atoms are too small to resolve (about the size of a grain of salt). The axon terminal is at the top and the muscle cell is at the bottom.



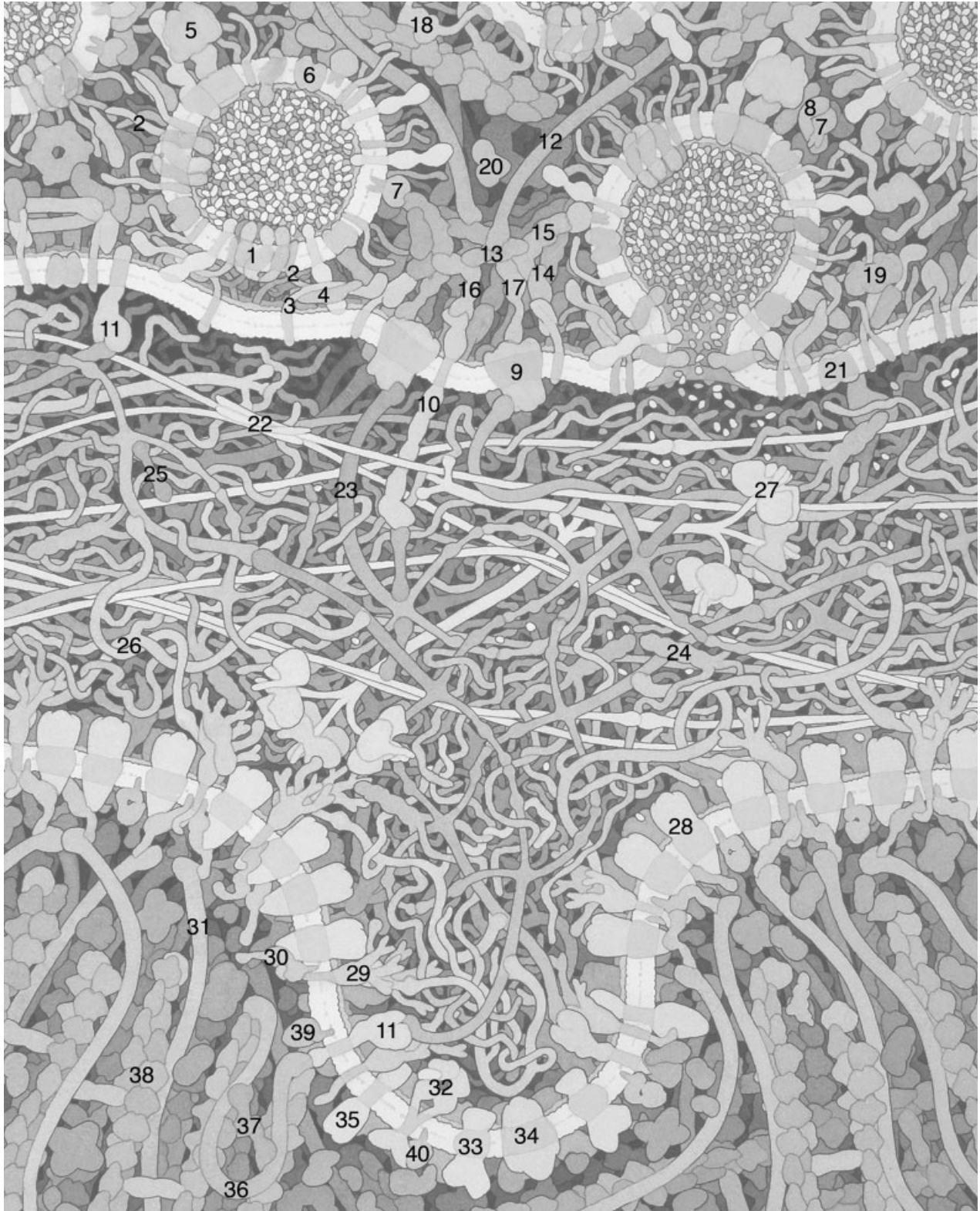


FIG. 3. **Key to Fig. 2.** Molecules in the vesicles include the following: 1, synaptophysin; 2, synaptobrevin; 3, syntaxin; 4, Snap25; 5, vacuolar ATPase; 6, acetylcholine transporter; 7, Rab3A; 8, GDI. The presynaptic active zone includes the following: 9, voltage-gated calcium channel; 10, LAR; 11, integrin; 12, bassoon; 13, CAST; 14, Munc-13/18; 15, RIM; 16, liprin; 17, MINT; 18, synapsin; 19, NSF; 20, choline acetyltransferase; 21, CHT1 sodium-coupled transporter. The synaptic cleft includes the following: 22, collagen IV; 23, laminin; 24, perlecan; 25, entactin/nidogen; 26, agrin; 27, acetylcholinesterase. The postsynaptic membrane and muscle cell include the following: 28, acetylcholine receptor; 29, dystroglycan; 30, rapsin; 31, utrophin; 32, ErbB receptor bound to neuregulin; 33, sodium-potassium pump; 34, voltage-gated sodium channel; 35, MuSK; 36, talin; 37, vinculin; 38, actin; 39, Src; 40, Ras.

protein ColQ [35]. The illustration here is based on the structure of acetylcholinesterase alone (PDB entry 1acj [36]) and models of the collagen tail [37, 38]. The ColQ tail is shown binding to perlecan. Estimates of the amount of acetylcholinesterase show up to 3,000 per square micron [31]. Assuming 12 active sites in each complex corresponds to a distance of about 60 nm between the complexes. I have included two in the illustration.

#### POSTSYNAPTIC STRUCTURE

In the course of researching this illustration, I came to a realization about cells, which in hindsight, seems obvious. The complex structures found in our cells must each be created, maintained, and regulated. As never before, researchers are discovering that there is an elaborate infrastructure that organizes everything. Thus, if a particular protein is found in a particular place, there is probably a structural protein that holds it there, and another set of proteins that regulate when it is put there.

The neuromuscular synapse is no exception. There is an elaborate infrastructure that creates the densely packed and closely opposed membranes of the synapse. The overall organization was taken from several schematic diagrams [39–44]. The structure of the acetylcholine receptor was taken from the crystal structure in PDB entry 2bg9 [45]. A complex assembly of proteins clusters acetylcholine receptors and links them to both the synaptic basal lamina and the cytoskeleton inside the cell. The membrane protein dystroglycan, which is composed of a transmembrane beta subunit and a synaptic alpha subunit, is at the center of this assembly. It binds to agrin on the synaptic side and to rapsin on the cytoplasmic side. Rapsin also has several binding partners. It is myristoylated at the N-terminal end, anchoring the protein to the membrane. It also has binding sites for itself, allowing it to self-associate into larger complexes, and binding sites for acetylcholine receptor and for utrophin [46]. The huge protein utrophin links the assembly to the cytoskeleton. This was based on the illustration of utrophin on the electron micrographic study of dystrophin, which shows a long, spectrin-like protein with a terminal globular domain [47].

Several proteins were included at the base of the fold. These include neuregulin and its receptor ErbB, with structure taken from a recent review [48]. The sodium-potassium pump is taken from the crystal structure in PDB entry 3b8e [49], and the voltage-gated sodium channel is taken from an electron micrograph reconstruction [50]. I have also included the MuSK (muscle-specific kinase), a transmembrane signaling protein [42], and an integrin complex. The integrin also links the basement membrane with the cytoskeleton, binding to laminin on the synaptic side, and talin and vinculin link the complex to the actin cytoskeleton inside the cell [51, 52]. The talin structure [53] and vinculin structure [54] are based on the results from the electron microscopy. I have also included several signaling proteins, including Src tyrosine kinase, based on the crystal structure in PDB entry 2src

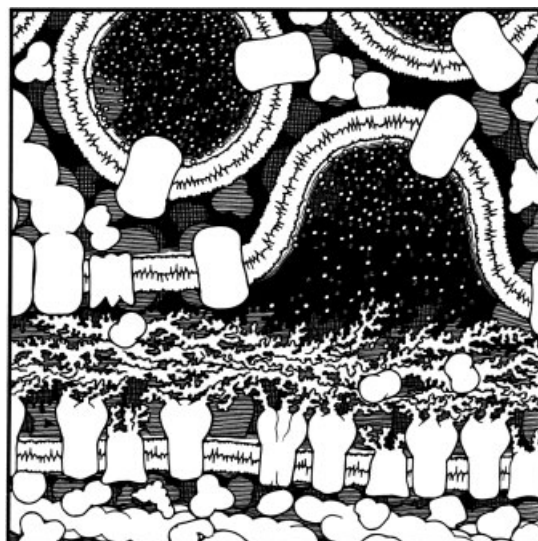


FIG. 4. Illustration of a neuromuscular synapse from the 1992 edition of the *Machinery of Life*.

[55], and Ras based on a crystallographic structure in PDB entry 1wq1 [56].

#### ARTISTIC CHOICES

When creating an illustration of a complex subject, many artistic choices must be made [57]. The synapse illustration combines several techniques to highlight the structural and functional features of the scene. First, I used a cross-sectional metaphor with very little perspective, instead of immersing the viewer inside the environment. This allows the display of a large section of the cell and allows all of the molecules to be drawn at the same scale, allowing easy comparison. The molecules are drawn with simple outlines and flat colors to highlight the packing and distribution of molecules instead of the details of each individual molecule.

The colors are chosen to highlight the ultrastructural arrangement of the synapse, with the cell cytoplasm in blues, the membranes and membrane proteins in green, and the synaptic space in earth tones. Intermediate colors are used for molecules that interact between these ultrastructural spaces—for instance, agrin, which links the postsynaptic membrane to the basement membrane, is colored as an earthy green, and synapsin and Rab3A, which link the synaptic vesicles to the cytoplasmic elements, are colored as blue-green.

#### PERSPECTIVE

We are currently at an exciting stage in the study of complex cellular structures. Researchers are inexorably characterizing each of the components of cells and assembling them in time and space within the context of the living cellular environment. With each passing year, this description becomes richer, filling in the unknown portions and discovering new (and often unexpected) avenues that still need to be explored.



Comparison of the synapse image from the 1992 edition of the Machinery of Life (Fig. 4) with the new illustration underscores some of these advances. The greater level of detail is, in large part, due to the abundant information that has become available in the last 15 years. However, it is also due to advances in information technology. In 1992, I relied on the citation index and review articles to move from topic to topic. Today, the fast search tools available through PubMed allow a broad and deep search of topics. This greatly facilitates integrative work such as the creation of these illustrations.

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