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Synaptogenesis

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INTRODUCTION

The study of synapse formation requires an understanding of synaptic function, structure, and organization. This chapter, therefore, reviews the essential roles played by synapses in the nervous system, the basic mechanisms of synaptic transmission, and the presynaptic and postsynaptic specializations that support synaptic signaling, before considering the events that establish, maintain, and modulate synaptic connections.

Synapses are arbiters of information flow in the nervous system. Information is carried through the nervous system by distinct intracellular and intercellular processes. Within neurons, information is encoded in the patterns of electrochemical activity that pass in waves across neuronal surfaces. Neuronal activity is then transferred between neurons by means of specialized intercellular signaling structures, the synapses. Synapses with non-neuronal targets such as heart and skeletal muscle regulate most bodily functions. The term *synapse*, from the Greek for "connect," intimates a close physical proximity between the synaptic specializations in adjoining cells. Indeed, we now know that where the speed and fidelity of synaptic communication is critical, presynaptic and postsynaptic specializations are directly apposed and precisely aligned (Fig. 1). Originally, however, Sherrington coined "synapse" in a physiology textbook in order to designate the functional linkage between neurons whose activities are coupled (Foster, 1897). Although an anatomical substrate for Sherrington's functional synapse was separately anticipated by others, including Cajal, Held, and Langley, the precise cellular arrangement at synapses remained uncertain until synaptic connections were finally observed in the electron microscope (De Robertis and Bennett, 1955; Palay, 1956).

Studies in succeeding decades revealed the basic mechanisms of synaptic signaling, or *neurotransmission*. Most synapses transmit neuronal activity by means of an intercellular chemical messenger, the *neurotransmitter*. Chemical neurotransmission begins as electrical activity in the presynaptic cell triggers the secretion of neurotransmitter (Fig. 2). The released neurotransmitter diffuses within the fluids of the extracellular space and ultimately binds to specific receptor proteins embedded in the surface membrane of the postsynaptic cell. Synaptic transmission is completed as changes in the conformation of the receptor induced by transmitter binding alters postsynaptic electrochemical activity. The chemical nature of neurotransmission was initially predicted from the effect of nicotine on neural transmission through peripheral ganglia. Nicotine was eventually shown to act as a specific ligand for a subset of the receptors for acetylcholine (ACh), the first neurotransmitter identified in the peripheral nervous system (PNS) and the central nervous system (CNS) (Loewi, 1921; Dale *et al*., 1936; Eccles *et al*., 1956).

Two broad functional classes of chemical synapse differ principally in their speed of neurotransmission. Fast chemical synapses are composed of closely opposed presynaptic and postsynaptic elements and typically employ *ionotropic* neurotransmitter receptors (Figs. 2 and 3). Ionotropic receptors are ion channels whose conductance is directly regulated by neurotransmitter binding. In skeletal muscles, for example, ACh released from motor nerve terminals allosterically opens cation-selective pores formed by the subunits of nicotinic ACh receptors (AChRs), which are concentrated on the surfaces of muscle fibers opposite the nerve. The resulting influx of cations is immediate and large and rapidly stimulates muscle activity. In contrast, presynaptic and postsynaptic specializations at slow chemical synapses, which are common in the autonomic innervation of glands and organs, are diffusely organized and often are not closely opposed to each other. Slow chemical synapses also often employ *metabotropic* receptors, which regulate cell function indirectly, through intracellular second messengers. Thus, in the heart, parasympathetic axons from the vagus nerve release ACh that activates metabotropic AChRs on the surface of cardiac myocytes. These AChRs are pharmacologically distinguished by their sensitivity to muscarine rather than nicotine. Activation of muscarinic AChRs indirectly opens cardiac potassium channels (Sakmann *et al*., 1983) through intermediary G-protein second messengers (reviewed by Brown and Birnbaumer, 1990). The resulting efflux of potassium from myocytes depresses cardiac excitability and gradually slows heart rate. Note that both the timing and strength of the response to ACh in cardiac muscle is muted compared to the immediate (millisecond), all-or-none contractile response in skeletal muscle. Regardless of synapse type and receptor mechanism, synaptic transmission ultimately ceases as transmitter is eliminated by re-uptake or catabolism, or the postsynaptic ion channels inactivate.

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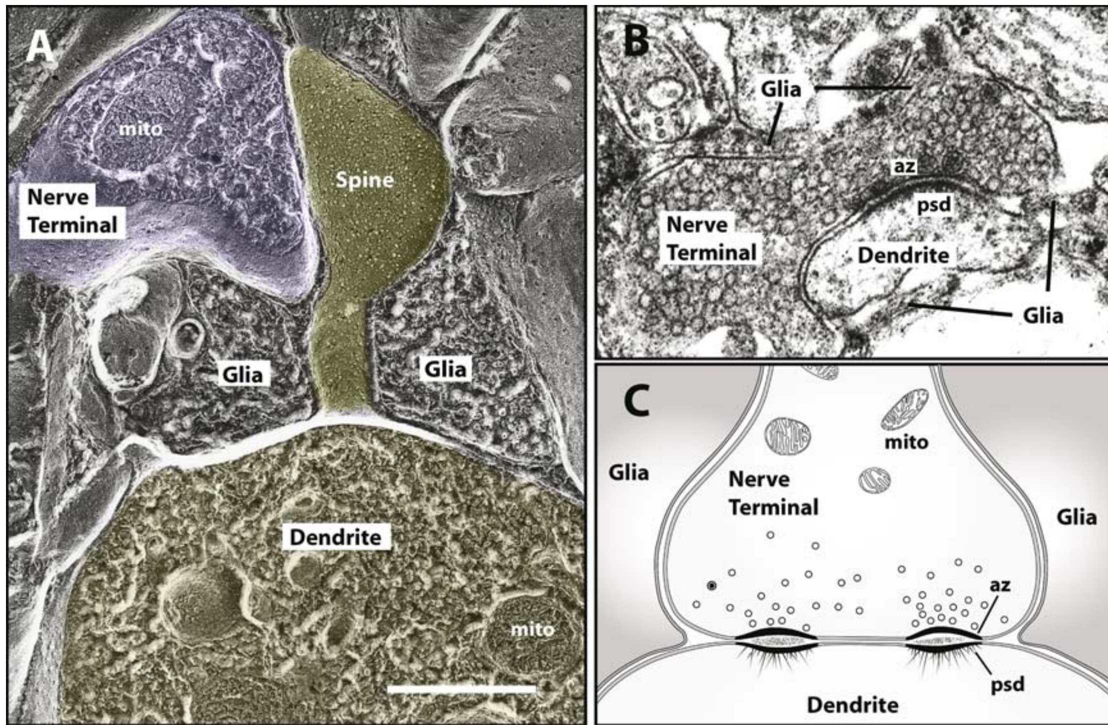


FIGURE 1. Cellular composition of the synapse. Synapses are specialized signaling structures assembled between neurons and their target cells for the accurate transmission of neural information. The location, speed, and strength of neurotransmission is dependent on the alignment of presynaptic specializations that control the secretion of neurotransmitter with postsynaptic specializations that transduce transmitter binding into changes in target cell activity. Synaptic features visible by microscopy include an enlarged presynaptic terminal (alternatively called a *bouton* or *varicosity*) containing mitochondria and high concentrations of small, clear "*synaptic vesicles.*" Nerve terminals at fast chemical synapses also contain *active zones* (az), membrane subdomains where transmitter secretion is enhanced; high concentrations of protein at active zones collect metal stains and appear dense in electron micrographs. The morphology of the postsynaptic cell often reflects the presynaptic terminal, and within the postsynaptic membrane, neurotransmitter receptors and signal transduction proteins are concentrated directly opposite the synaptic cleft from transmitter release sites. Glial cells typically surround synapses and provide metabolic support. (A) Scanning electron micrograph of a spine synapse on a pyramidal cell in the hippocampus of an adult rat, revealed by freeze-fracture methods. Spines are short protrusions from dendritic shafts, an anatomical arrangement which partially isolates many of the synaptic inputs to a single dendrite. Image kindly provided by Tom Reese; N.I.H. (B) Transmission electron micrograph of a synapse in the superior cervical ganglion of an adult mouse. Typical of many chemical synapses, the presynaptic terminal contains many clear synaptic vesicles concentrated opposite a dense region of postsynaptic membrane (the postsynaptic density, or PSD). Biochemical and immunochemical studies reveal PSDs are rich in cell adhesion proteins, transmitter receptors, and receptor-associated scaffolding proteins. Typical of excitatory synapses, the nerve terminal also contains a few dense core vesicles, which contain neuromodulatory peptides and/or components of the synaptic cleft, and a cluster of vesicles associated with a dense region of presynaptic membrane, known as an active zone (az). Notably, active zones and PSDs are precisely aligned. Most nerve terminals also have several mitochondria, not visible in this section. (C) Model chemical synapse, containing adherent pre- and postsynaptic elements with aligned sites of transmitter release and transmitter reception, surrounded by glial cells.

The properties of fast chemical synapses in particular have evolved beyond a simple means of exchanging neural information, to facilitate higher neural functions. By controlling the *timing*, the *location*, and the *strength* of neurotransmission, synapses act as gates to the flow of neural activity through the brain and body. Therefore, in most organisms, synaptic transmission is also a primary site of modulation of neural information. The strength and disposition of synaptic connections within the neural architecture so critically determine overall neural function that they comprise a secondary mechanism of encoding neural information. That is, an ability to change synaptic strength and location are most likely the biochemical and cellular substrates of learning and memory.

Fast synaptic transmission is promoted by an elaborate series of cellular and molecular mechanisms, which will remain the focus of this chapter. Neurotransmission is chiefly controlled at the steps where electrical and chemical signals are interconverted. Perhaps this should not be too surprising. The interconversion of electrochemical (ion flux) and chemical (transmitter) activity levels are the most complicated biochemical steps in the flow of neural information; many cellular processes are most heavily regulated at their slowest and most complex steps. The *timing* of neurotransmission is precisely controlled by tightly coupling presynaptic depolarization to neurotransmitter secretion. Coupling occurs through the use of calcium as a trigger for secretion, and by concentrating voltage-sensitive calcium channels at synaptic sites. *Location* is specified by tightly focusing neurosecretion and neuroreception at small sites on the pre- and postsynaptic cell surfaces. Importantly, these specialized signaling domains are co-localized at sites of adhesion between the

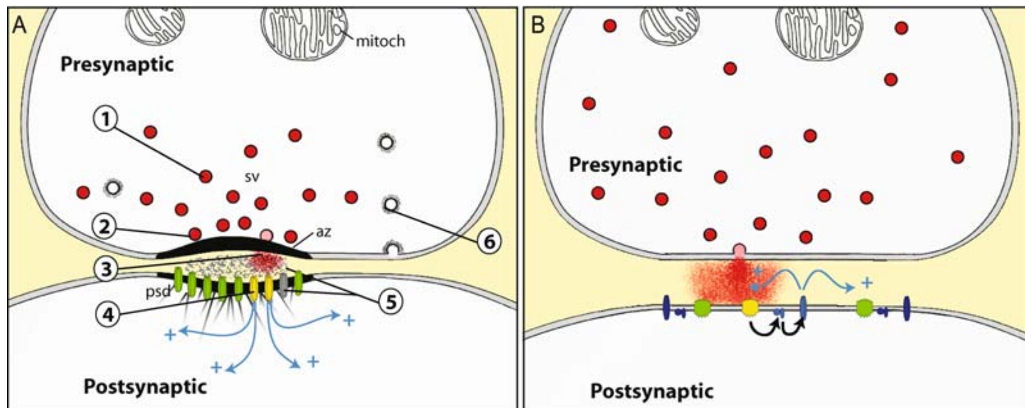


FIGURE 2. Neurotransmission. (A) (1) Neurotransmitter is initially concentrated in small lipid-walled vesicles within the presynaptic terminal. (2, 3) Transmission begins as presynaptic depolarization triggers the fusion of one or more synaptic vesicles with the synaptic membrane of the nerve terminal. Released transmitter diffuses across the synaptic cleft. (4) Binding of neurotransmitter to specific receptors in the postsynaptic membrane directly or indirectly changes the activity of postsynaptic ion channels. For example, excitatory transmitters such as glutamate open cation-selective ion channels and depolarize the postsynaptic cell. (5) Transmission ends as transmitter-induced currents are inactivated, either through clearance of transmitter from the synaptic cleft, or through biophysical properties intrinsic to the receptor or ion channels. (6) Excess presynaptic membrane is removed by endocytosis. At fast chemical synapses, pre- and postsynaptic specializations are directly apposed and precisely aligned. (B) In contrast, pre- and postsynaptic elements are loosely associated and minimally organized at slow chemical synapses. For example, presynaptic membranes lack active zones, and postsynaptic membranes have low concentrations of transmitter receptors. Transmission at slow synapses often relies on metabotropic receptors, which indirectly regulate membrane conductance through secondary messengers.

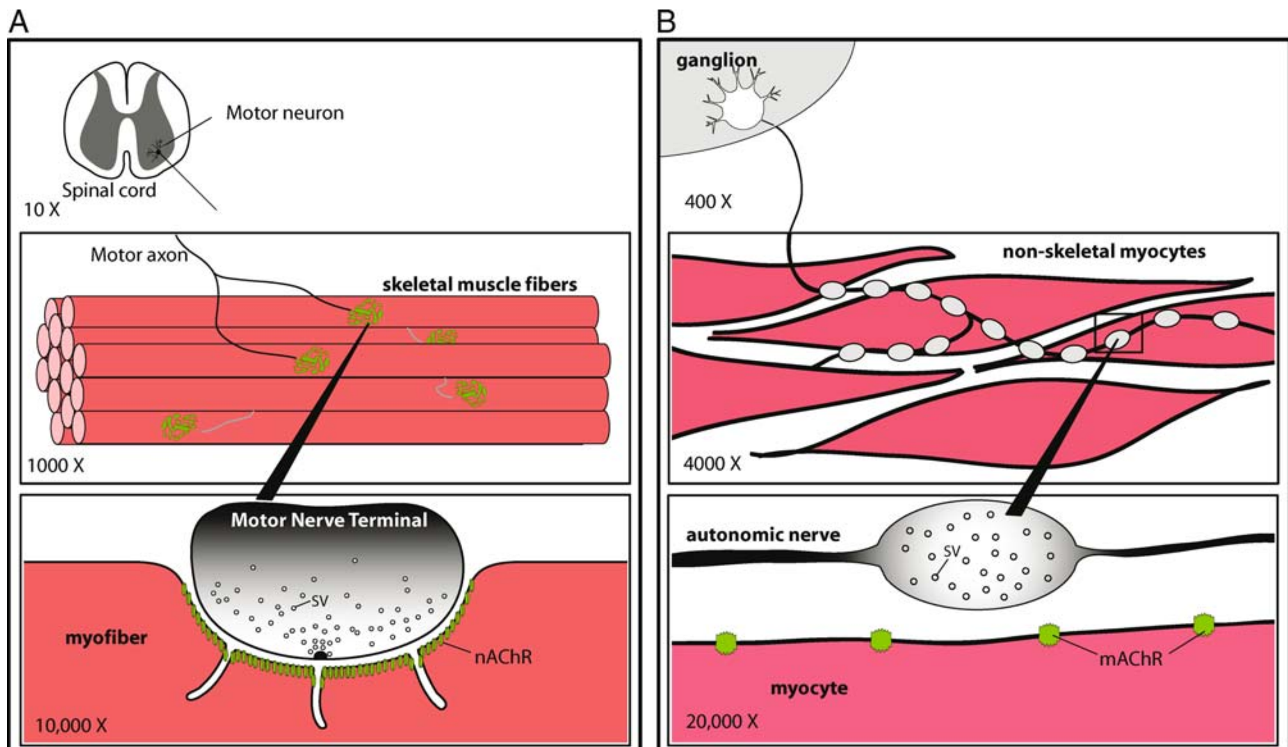


FIGURE 3. Prototypical fast and slow chemical synapses in muscle. Skeletal and cardiac muscles both receive cholinergic innervation, but each contains a different type of chemical synapse. (A) In skeletal muscle, axons from spinal motor neurons form fast chemical synapses at specific sites on each muscle fiber. Motor nerve terminals are located precisely opposite high concentrations of nicotine-sensitive ionotropic ACh receptors (nAChR) in the muscle fiber surface. Within the motor terminal, synaptic vesicles are polarized towards the synaptic surface, and further concentrated near active zones. (B) In cardiac muscle, sympathetic axons contain presynaptic varicosities which are widely distributed and which lack polarity or active zones. Cardiac myocytes contain metabotropic ACh receptors (mAChR), which are not concentrated near axons, and which are indirectly coupled to cardiac potassium channels through G-proteins. Approximate scales are provided at lower left corners of each figure.

axon and its target cell. Precise spatiotemporal control distinguishes synaptic transmission from the diffuse chemical signaling that coordinates the metabolic activity of an animal. Finally, the *strength* of synaptic transmission is dependent on the amount of neurotransmitter secreted in response to presynaptic activity, and the size of the postsynaptic response to a given amount of transmitter.

Fast chemical synapses have three cellular elements. First, the nerve terminal of the presynaptic cell contains specialized neurosecretory domains, which regulate the timing, location, and volume of neurotransmitter release. Since neurosecretion typically occurs far from the nucleus of the cell, presynaptic specializations also include mechanisms to locally synthesize and package transmitter into vesicles, and to recover synaptic vesicle materials following release. Second, the surface of the postsynaptic cell is specialized to recognize secreted neurotransmitter, and to transduce the chemical energy of binding into altered electrical activity. Excitatory transmitters often increase depolarizing conductances, as just described for ACh at the neuromuscular synapse. However, many variations on this mechanism have evolved. For example, neurotransmitters at some sensory synapses alter postsynaptic activity by closing ion channels. Third, most synapses are enshrouded by glial cell processes. Glial cells play important roles in supporting the metabolic activity of the pre- and postsynaptic elements. They also strongly influence the potential for growth and synaptogenesis by axons and dendrites.

Perhaps most importantly, fast synapses are sites of direct contact between the pre- and postsynaptic cells. The precise pairing of pre- and postsynaptic specializations is so fundamental to fast chemical neurotransmission that it may at first appear trivial. In fact, proximity is an essential mechanism underlying the speed and specificity of synaptic signaling and has profound consequences for neural function. A narrow synaptic cleft between the sites of transmitter release and reception means the neurotransmitter will diffuse only a few dozen nanometers to complete transmission. Just as importantly, restriction of synaptic transmission to small domains allows information to be distributed to specific subsets of cells and specific portions of those cell's surfaces, rather than willy-nilly between all potential matches. One consequence is that synapses often grossly outnumber the cell bodies they connect (Fig. 4). The resulting convergence and divergence of interneuronal signaling enables the nervous system to process and integrate information rather than merely relay it. A second consequence is that patterns of neural connectivity can be modified without wholesale cellular restructuring of the brain, by altering individual synaptic elements.

The coordinated assembly of pre- and postsynaptic specializations constitutes *synapse formation*. An initial phase of synaptic development establishes a general pattern of innervation, in which specific sets of cells are connected. The initial synaptic connections are then remodeled. Synaptic reorganization is influenced by fluctuating levels of activity among subsets of connections within the architecture, as well as by circulating humeral factors. In response to differing levels of activity, some synapses are selectively strengthened and maintained, while others are simultaneously eliminated. The overall effect is one of progressive restriction, narrowing initially broad patterns of innervation into functionally refined subpatterns. In some organisms, activity-dependent refinement of synaptic connections completes neural development. In many, however, the remodeling phase of neural development melds with processes of learning and memory and continues throughout life. A critical feature of vertebrate neural systems is that the capacity for computation, adaptation, and fine control in the adult animal depends as much on the specificity and plasticity of the synaptic connections as on the number of connected elements.

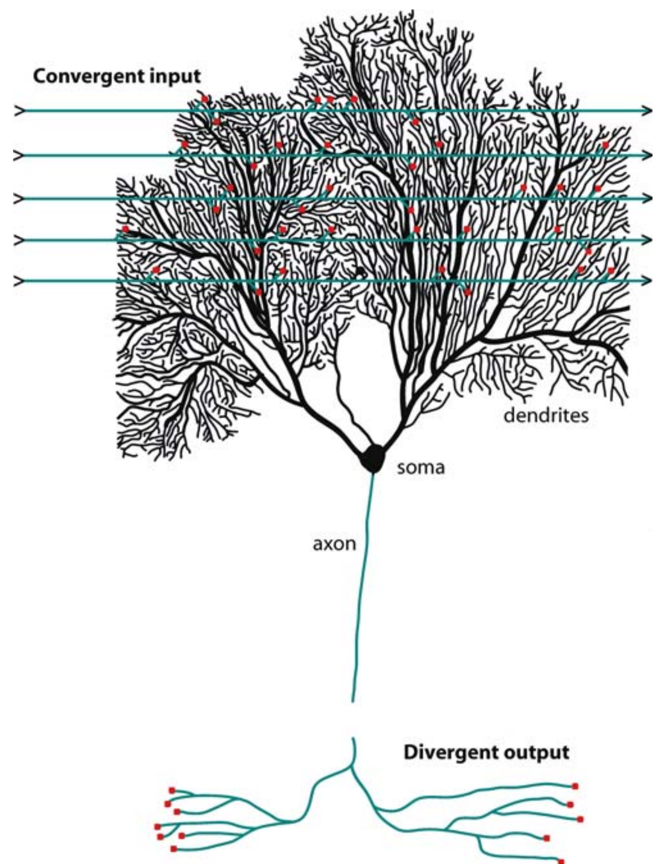


FIGURE 4. Convergence and divergence of neural information. Most neurons contain an array of dendrites, which receive hundreds of synaptic inputs. Dendritic processes ultimately converge at or near the neuronal soma. Conversely, a single axon typically emerges from the soma before branching to innervate many target cells. Synaptic activity at single dendritic sites is generally insufficient to bring the axon to the threshold of an action potential. Thus, activity in the axon represents the integrated synaptic activity in the dendritic arbor. The convergence and divergence of synaptic inputs allows neuronal systems to process information.

In principle, the precise colocalization of pre- and postsynaptic specializations could arise through cell-autonomous programs of development. Indeed, most synapsing cells independently express their synaptic components and can assemble functional pre- or postsynaptic elements alone, in the absence of a synaptic partner. Nevertheless, most synapse formation involves

the coincident assembly of new pre- and postsynaptic specializations at sites where the two cells make contact. This indicates that neurons and their targets exchange *synaptogenic signals*, and that these signals act locally to promote the assembly of synapses from already synthesized components. In short, synapses are organized structures rather than induced programs of development.

THE NEUROMUSCULAR JUNCTION: MODEL SYNAPSE

Much of our understanding of synaptic organization is derived from studies of innervation in the skeletal muscles of vertebrate animals. Synapses between motor axons and muscle fibers are known as neuromuscular junctions (NMJs). Historically, innervation in muscle presented clear advantages for experimentalists. Compared to most interneuronal synapses, skeletal NMJs are large and physically isolated from each other (Fig. 5). They are also physiologically robust. The accessibility of this preparation led to the experiments that defined and confirmed the existence of chemical neurotransmitters, the vesicular hypothesis of neurotransmitter release, and the principal mechanisms of postsynaptic excitation.

An apparent disadvantage of NMJs is that they account for only a tiny fraction of the mass of a muscle. Ordinarily, this would prevent a straightforward analysis of the biochemical constitution of this synapse. Instead, biochemistry has been one of the NMJ's great advantages, due largely to an ontogenic relationship between the skeletal NMJ and the electric organ structures present in certain species of fish, such as the marine ray *Torpedo* (see Box 1). Fractionation of the electric organ led to the discovery of a number of key synaptic components. Some, like VAMP (vesicle associated membrane protein), turned out to be important components of virtually all chemical synapses; VAMP was later independently identified as synaptobrevin, in synaptosomal fractions of homogenized bovine brain. Others components were more specific to the neuromuscular synapse. For example, the nicotinic AChR was the first neurotransmitter receptor (in fact, the first ion channel) to be molecularly characterized and cloned, due to its enrichment in electric organ membranes (Schmidt and Raftery, 1973; Noda *et al*., 1982; Claudio *et al*., 1983; Numa *et al*., 1983). Another important example is agrin, the first synaptic organizing signal to be molecularly identified, which was also purified from Torpedo electric organ homogenates (Nitkin *et al*., 1987). As a result, a good deal is known about how motor neurons direct synapse formation in skeletal muscles (described in a later section). In contrast, the identification of molecules that distinguish and organize the various types of chemical synapses in the brain has lagged, in no small measure because a homogeneous population of central synapses amenable to biochemistry has not been available. Instead, brain has proved to be good starting material for the identification of ubiquitous synaptic components. For example, SNAP-25, syntaxin, synapsin, synaptophysin, and munc18 are an ancient retinue of proteins discovered in extracts of mammalian brain that regulate presynaptic vesicle dynamics in nerve terminals throughout the body, in animals throughout the phylogenetic tree.

A further property of the neuromuscular system especially useful to developmental neurobiologists is that much of it is capable of regeneration. The ability of peripheral nerves and skeletal muscle fibers to regenerate has allowed processes of synapse assembly at the NMJ, which begins prenatally in mammals, to be reassessed following injury in adults. As a direct result, studies of reinnervation in skeletal muscle have played key roles in formulating and testing three fundamental concepts in neuroscience. The first is the essential notion that the synapse is the site of communication between nerves and their targets, which developed by the beginning of the last century. Second is the concept of synaptic specificity in neural development, as motor axons were found to reinnervate very specific sites on muscle fibers by Cajal and his students. Third is the molecular basis of synapse formation, conceived by Cajal early in the last century and pursued into the current one.

The NMJ possesses two final advantages for the current generation of neuroscientists. First, molecular information

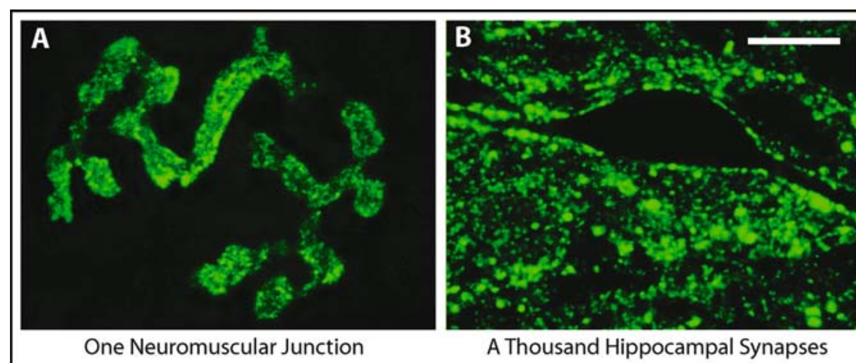
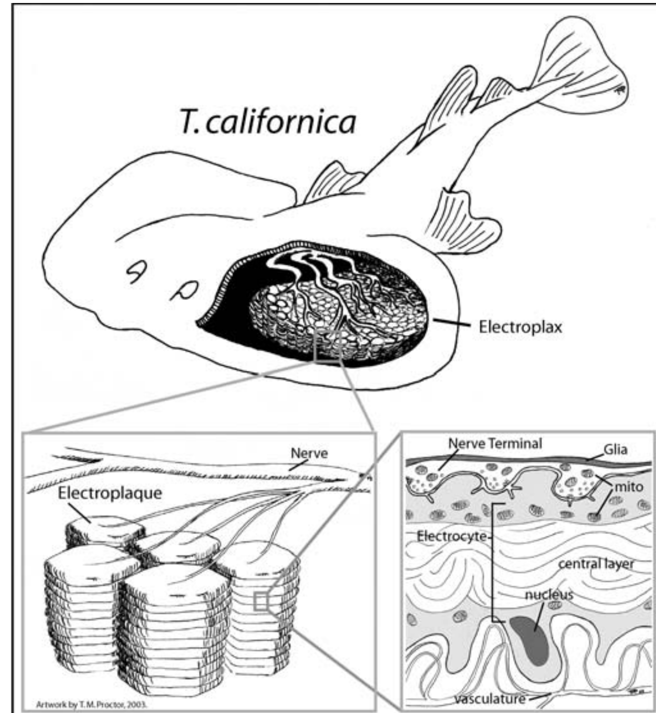


FIGURE 5. Neuromuscular synapses are much larger than most interneuronal synapses. (A) Motor nerve terminal at a single skeletal neuromuscular junction from an adult mouse. (B) Several hundred nerve terminals in the CA3 region of the hippocampal formation from a juvenile rat. Confocal images at similar scales show immunoreactivity for synapsin.

BOX 1. The Swimming Purified Acetylcholine Receptor



Strongly electric fish, including the Torpedinidae family of marine rays, contain specialized electrogenic organs. The Pacific marine ray (*Torpedo californica*) pictured above-left often reaches a meter in width and is capable of generating 50–100 V discharges. *T. cal*'s electric organs, or electroplax, generate moderate pulses as a defense against faster swimming predators such as sharks, and strong discharges to immobilize fast-swimming prey like salmon. Charles Darwin considered it "impossible to conceive by what steps these wonderous organs have been produced" (Darwin, 1981). Actually, the bilateral kidney-shaped organs are embryologically derived from the branchial musculature. Embryonic myotubes lose their skeletal muscle myosins and collapse longitudinally to form electroblasts. Electroblasts spread horizontally and intercalate to form stacks of disc-like differentiated electrocytes. One entire face of each electrocyte is then innervated by motor axons, but always on the same side (dorsally in *T. cal*), which orients all electrical activity in the same direction. Neural stimulation depolarizes the postsynaptic membrane of the electrocyte, producing an immediate 100 mV charge reversal. The electrocyte's central layer, which is a remnant of the sarcomeres, may transiently insulate the opposite side of the cell, polarizing the overall current flow. The depolarization of each electrocyte in the stack is synchronized through coordinated neural stimulation; their summed discharges peak at over 50 V, and repeat at more than 400 Hz, enough to shock any adjacent sea creatures into compliance (either submission or avoidance). Thus, the voltage-generating electroplaques are hugely overgrown neuromuscular junctions, piled in series like a (very) tall stack of pancakes (above). There are typically about 400 electroplaques in each voltaic stack, and up to 400 stacks in each organ, which together make up to 30% of a ray's body mass. This represents an extraordinary (possibly even shocking) abundance of AChR-rich postsynaptic membrane in a tidy package. Indeed, to biophysicists and neurobiologists, "The torpedo ray … is essentially a swimming purified acetylcholine receptor" (Miller, 2000). (Photo by Howard Hall, used with permission; original artwork by Thomas M. Proctor.)

gained over the last several decades now permits sophisticated, mechanistic questions about synapse formation to be addressed. Second, the size, isolation, and regenerative capacity which attracted early students of the nervous system remain a distinct advantage to the advanced imaging methods that are now beginning to reveal the cellular and molecular dynamics involved in synapse formation and plasticity.

Innervation and Transmission in Muscle

A general principle of synaptic transmission at the vertebrate skeletal NMJ is that patterns of impulses in the nerve are highly correlated with contractile activity in the muscle. Reliable coupling of nerve activity to muscle activity ensures that, given adequate stimulation, every fiber in the muscle can be recruited to heroic efforts, be it the sprint of a fieldmouse evading a hawk, or the strain of a paleo-hunter throwing a spear. Yet, in both predator and prey, the very same synaptic connections may also be employed in the performance of finely graded tasks. By moderating neural activity, the strength and timing of muscle activity can be exquisitely controlled to effect the fluid stroke of a cheek or a pen, the accurate movements in a throw and a catch, and the intricate labial, lingual, and laryngeal sequences of speech.

Two general features of neuromuscular innervation underlie the simultaneous robustness and fine control of neuromuscular coupling. First, the strength of individual synaptic connections in muscle is extraordinarily high. Second, each muscle fiber is innervated by a single motor axon, and each motor axon innervates a discrete number of muscle fibers. A single motor axon and the several muscle fibers it innervates are termed

a *motor unit*. Combining these features ensures that a nerve impulse always generates a response in the muscle, but that the size of the response can be scaled, depending on how many motor neurons are activated, and the size of the motor units recruited. We consider these mechanisms in turn.

Synaptic Efficacy at the NMJ

Synaptic transmission at the NMJ rarely fails. A single action potential in the motor axon will ordinarily elicit a synaptic event capable of depolarizing the postsynaptic membrane in the muscle fiber to nearly 0 mV, which is well beyond the threshold for action potential propagation along the muscle fiber. The synaptic strength required to achieve the high fidelity of neuromuscular transmission is considerable. Not only must the postsynaptic current be large enough to overcome the low-input resistance that comes with the large diameter of the muscle fiber (often 50 μm), but signaling in most muscles occurs at levels that are several-fold above the minimum needed to gain full response to a single nerve impulse. In many muscles, more than 80% of the junctional receptors can be blocked before the muscle's response is detectably diminished (Fig. 6). This apparent excess capacity for transmission ensures that nerve and muscle activity remain tightly coupled during periods of intense demand and is known as the *safety factor* (Wood and Slater, 2001). The strength of a synaptic connection is a function of the amount of neurotransmitter secreted by the presynaptic cell in response to depolarization, and the amount of depolarization that occurs in the postsynaptic cell in response to neurotransmitter. A high safety factor for transmission depends in addition on specializations that sustain high levels of transmitter release and large postsynaptic responses during repetitive stimulation. These include both chemical and structural mechanisms, outlined below.

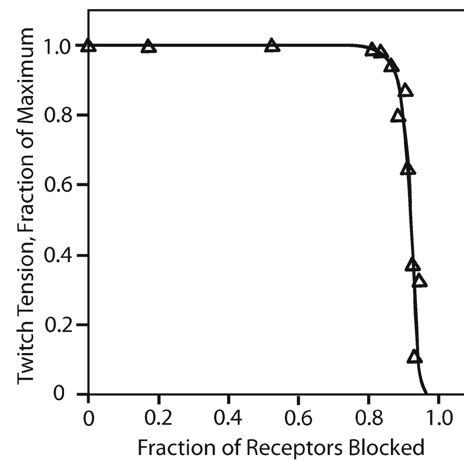


FIGURE 6. Safety factor for neurotransmission. At some synapses, the strength of neurotransmission dramatically exceeds the level required to guarantee a full postsynaptic response to evoked release of transmitter from the presynaptic terminal. For example, more than 80% of ACh receptors at the neuromuscular junction may be blocked by pharmacological antagonists before muscle contractions elicited by stimulation of the motor nerve are noticeably weakened. Thus, the safety factor for solitary synaptic events at the vertebrate neuromuscular junction is usually greater than 5-fold and may exceed 10-fold in muscles such as the diaphragm which are especially resistant to inhibition. The apparent excess signaling capacity is known as the safety factor in neurotransmission. It permits high fidelity neurotransmission to continue during periods of intense demand. The safety factor for neuromuscular transmission is significantly reduced in patients with the autoimmune disorder *myasthenia gravis*, in which antibodies to the muscle ACh receptor impair postsynaptic responsiveness. The extraordinarily high safety factor in diaphragm muscles allows neuromuscular blockers to be used in clinical care, as their proper titration will relax airway, limb, and axial muscle relaxants do not arrest breathing. In addition to levels of postsynaptic receptors, a high safety factor depends on elevated levels of neurotransmitter release from the presynaptic terminal, efficient coupling of transmitter binding to postsynaptic activity, and rapid clearance of spent neurotransmitter from the synaptic cleft.

Presynaptic Mechanisms

The motor nerve terminal is highly specialized to promote and sustain high levels of neurotransmission. First, the extraordinary size of each motor nerve terminal (Fig. 5) accommodates hundreds of *active zones*, specialized membrane domains where neurotransmitter is preferentially released. Second, like other fast chemical synapses, motor terminals are specialized to speed both the release of neurotransmitter and the reconstitution of new transmitter-laden synaptic vesicles (Fig. 7).

Synaptic vesicles are initially concentrated near release sites along the presynaptic membrane. The molecular mechanisms that polarize the distribution of synaptic vesicles near release sites have not been confirmed, but likely depend in part on interactions with the actin cytoskeleton that permeates the terminal. Additional interactions with components of the active zone complex then recruit synaptic vesicles to docking sites along the terminal surface membrane. Vesicle docking is mediated by a SNARE complex, which includes the vesicle membrane protein VAMP/synaptobrevin and the plasma membrane proteins SNAP-25 and syntaxin. Docking effectively primes a subset of synaptic vesicles for immediate release. The SNARE complex also drives the fusion of vesicle and terminal surface membranes, but only when appropriately triggered (Sollner *et al.*, 1993). The trigger for fusion is calcium. Intracellular levels of calcium are maintained at very low concentrations in resting nerve terminals, but rise sharply upon depolarization of the nerve terminal membrane from influx through voltage-dependent calcium channels in the terminal surface. The voltage-dependence of the presynaptic calcium channels is critical; they open only when the nerve terminal membrane is strongly depolarized. The proposed calcium sensor is the calcium-binding protein synaptotagmin, which is concentrated in synaptic vesicle membranes. Calcium entry into the terminal is concentrated at vesicle docking sites by recruiting calcium channels to active zones, through interactions with presynaptic membrane proteins such as syntaxin. (In fact, it is entirely possible that active zone complexes are recruited to the location of calcium channels, which may themselves be anchored to extracellular substrates.) Together, these multiple features ensure that neurosecretion is targeted to specific sites on the neuronal surface, and tightly coupled to axonal activity.

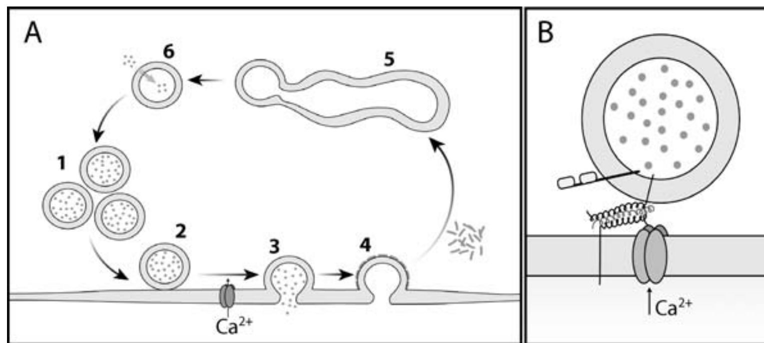


FIGURE 7. Synaptic function depends on regulated trafficking of synaptic vesicle components. Synaptic terminals far from the cell body employ signaling components that are locally synthesized or reused. Primary neurotransmitters are therefore simple biomolecules, such as amino acids or their metabolic relatives, and synaptic vesicles are reconstituted following synaptic activity. (A) The synaptic vesicle cycle. Prior to release of neurotransmitter, synaptic vesicles are concentrated near the synaptic surface of the nerve terminal (1), and dock at sites along the presynaptic membrane (2) through direct or indirect interactions with Ca^{2+} channels. Vesicle and surface membranes fuse in response to elevated intracellular Ca^{2+} concentrations following an action potential, releasing transmitter into the synaptic cleft (3). Vesicle membranes and proteins are internalized through clathrin-mediated endocytosis (4) at sites adjacent to the sites of fusion, and traffic through endosomal intermediates (5) before reforming small, clear synaptic vesicles. New vesicles are reloaded with neurotransmitter (6), by transporters powered by a pH gradient across the vesicle membrane. (B) Molecular model of vesicle docking. Docking is ultrastructurally defined by direct apposition of vesicle and plasma membranes, physiologically characterized by fusion in response to osmotic shock, and biochemically mediated by the formation of a SNARE complex. Synaptic vesicles contain the V-SNARE VAMP (synaptobrevin). Terminal membranes contain the target membrane T-SNAREs syntaxin and SNAP-25. Direct interactions between α-helical domains in each V- and T-SNARE produce a coiled-coil structure that holds vesicle and plasma membranes close together. Secondary interactions mediated by syntaxin link vesicles to voltage-activated Ca^{2+} channels. Synaptotagmin in the vesicle membrane likely mediates Ca^{2+}-induced fusion of vesicle and plasma membranes. Ca^{2+} binds to synaptotagmin at a pair of C2 domains, regulatory motifs first identified in the lipid- and Ca^{2+}-activated enzyme protein kinase C. Membrane fusion may be driven by conformational changes in the SNARE complex coiled-coil. How synaptotagmin drives fusion remains controversial.

The close proximity between sites of calcium entry and vesicle docking provides a three-fold benefit to rapid neurotransmission at the NMJ. First, the speed of neurosecretion is maximized by minimizing the delay between terminal membrane depolarization and vesicle fusion. Second, neurosecretion is topographically focused, as calcium levels rise fastest and highest where the channels are concentrated. Third, and for similar reasons, neurosecretion is chronologically focused and therefore synchronized at active zones throughout the motor terminal. Thus, active zones and calcium channels play a central role in fine-tuning the location and timing of neurosecretion. The concerted regulation of both the timing and location of transmitter release by calcium may ensure that depolarization does not cause release from errantly docked vesicles, and that properly docked vesicles release transmitter only in response to depolarization.

Postsynaptic Mechanisms

The postsynaptic region of the muscle fiber is called the *endplate*. The endplate's response to secreted neurotransmitter is determined principally by features of the nicotinic AChR. First, as described above, nicotinic AChRs directly translate neurotransmitter binding into membrane depolarization. The nicotinic AChR is composed of five homologous transmembrane subunits with a stoichiometry of 2α, 1β, 1γ, and 1δ. These are assembled as a ring around a membrane-spanning pore, which remains closed in the absence of ACh. By binding to specific sites on the extracellular surface of the ring, ACh allosterically opens, or "gates," the central cation-selective pore: Upon binding, the channel bends slightly, the pore opens, and Na^{+} (and some Ca^{2+}) ions flood into the muscle fiber. Importantly, the rate at which AChR channels open after ACh binds does not delay neurotransmission. A second way in which AChRs promote a postsynaptic response is through their relatively high ionic conductance, which speeds depolarization of the muscle fiber. Third, AChR channels close immediately upon ACh dissociation, but do not inactivate, and desensitize only slowly. Neurotransmission is therefore highly correlated to levels of ACh in the synaptic cleft. Fourth, the density of AChRs is maintained at extraordinarily high levels in the portion of the muscle membrane immediately subjacent to the nerve terminal (Fig. 8). The high density of transmitter-activated ion channels provides the endplate with the capacity to generate large postsynaptic currents in response to high levels of transmitter released from the nerve terminal. As discussed below, the clustering of transmitter receptors opposite the nerve terminal and the formation of a nerve terminal directly opposite clustered receptors are the fundamental events in the construction of a synapse.

One final high-performance feature of the nicotinic AChR is that channel activation is cooperatively dependent on ligand binding. The AChR channel opens only after two ACh molecules have bound. This makes it unlikely that low levels of ACh in the synaptic cleft will depolarize the postsynaptic membrane and thus improves the fidelity of neurotransmission by suppressing false alarms. The spontaneous release of neurotransmitter from the terminal is also suppressed, by mechanisms that remain incompletely understood, but which are likely to be directly

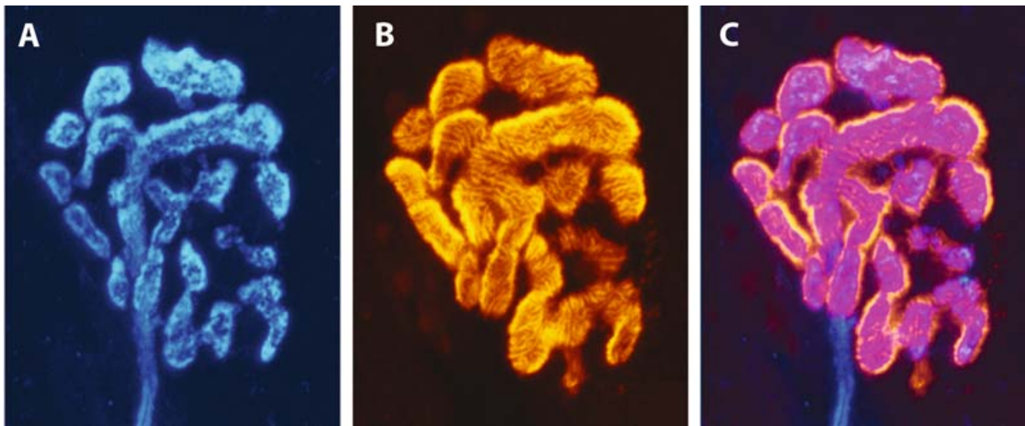


FIGURE 8. Morphological differentiation at the vertebrate neuromuscular junction. Staining skeletal muscle with antibodies to motor axons (neurofilaments and synaptophysin) and α-bungarotoxin, which binds tightly to ACh receptors, reveals that motor nerve terminal and endplate have matching conformations. In these confocal images of an adult mouse neuromuscular junction, antibody (blue) and toxin (yellow) were pseudocolored to visualize differences and overlap between nerve terminal and endplate. (A) The motor nerve terminal is branched and varicose compared to the pre-terminal axon. (B) ACh receptors are highly concentrated in postsynaptic membranes. Striations in toxin staining reveal the orientations of postsynaptic folds. (C) Pre- and postsynaptic domains show almost complete overlap.

incorporated into the transmitter-release machinery. These combined pre- and postsynaptic mechanisms not only reduce background noise in synaptic transmission, but also sharpen the postsynaptic response during *bona fide* synaptic events.

Single Innervation of Muscle Fibers

A second critical feature of neuromuscular innervation is that each muscle fiber is innervated by a single motor axon (Fig. 8). This facilitates control over the strength of muscle contraction. Other examples of monosynaptic input include the primary auditory relay synapse in the brain (the calyx of Held), and innervation in some autonomic ganglia, where information is rapidly and topographically exchanged without significant editing. In contrast, most neurons receive hundreds of convergent excitatory inputs, whose summary activity is required to depolarize the cell above threshold. Compared to the highly convergent and divergent patterns of innervation that complicate synaptic architecture in the CNS, innervation of muscle fibers appears simple. Indeed, the relative simplicity of muscle innervation has seduced several generations of neuroscientists, starting with Ramon y Cajal.

However, the simplicity of muscle innervation is deceptive. First, the exact match of innervating nerve terminals to muscle fiber number means that mechanisms of neuromuscular development must ensure complete innervation without polyinnervation. Stochastic methods of randomly plugging axons onto muscle fibers would leave some fibers without innervation, and some with more than one input. (Consider the likely result of playing 36 trials on a 36-slot roulette wheel.) Alternatively, it may be imagined that proper innervation would be most accurate if it followed a predetermined program of innervation, in which each neuron was genetically and hence, biochemically matched to a particular muscle fiber, perhaps through specific cell-surface recognition molecules. In short, motor units would be molecularly defined. Evidence for a high level of cellular determinism has in fact been found in the neuromuscular systems of invertebrate animals, as in the genetic model organisms *Caenorhabditis* and *Drosophila*. In vertebrates, axon outgrowth is directed to target fields (see Chapter 9), and individual pools of spinal motor neurons innervate specific muscles groups. However, synapse formation in vertebrate muscles appears considerably less determined at the level of individual muscle fibers. For example, motor nerves still fully innervate skeletal muscles when the size of the target muscle is experimentally increased, or the pool of motor neurons is decreased. In adult muscles too, partial denervation leads to an increase in the size of the remaining motor units, as uninjured axons sprout collateral branches that innervate the denervated portion of the muscle. We understand, therefore, that muscles interact with their innervating population of motor axons, influencing their growth and propensity to form synapses. Muscles must supply signals that both promote and retard synapse formation in the nerve.

A second complication is that individual muscles usually contain a mixture of muscle fiber types, which are selectively innervated by subtypes of motor axons. Individual muscle fibers differ in their complement of myosin isoforms and levels of glycolytic enzymes—factors that determine the rates at which the fibers contract and subsequently fatigue. Similarly, motor axons supplying a given muscle vary in diameter, conduction velocity, and nerve terminal structure. In birds and mammals, which are best studied, direct mapping of connections and detailed recording of muscle contractions during graded stimulation of the nerve have shown that muscle fibers are not randomly assigned to motor units. Instead, motor units contain primarily muscle fibers of a similar type (fast- or slow-twitch; high- or low-activation threshold). Motor units also vary considerably in size, as each

motor axon supplying a given muscle branches to innervate from tens to hundreds of muscle fibers. Large motor axons synapse with a greater number of large, more forceful muscle fibers. Small caliber motor axons innervate smaller groups of weaker, slow-twitch fibers. The resulting functional diversity in motor unit physiology enables individual muscles to shift among different use patterns by progressively activating motor units with differing contractile properties through increased levels of activity in the nerve. Progressive stimulation of larger proportions of a target cell population is called *recruitment*.

The selective organization of specific axons and muscle fiber types into particular motor units is one demonstration that the specificity of innervation extends to subtle differences among cells within a target population. Studies have established that one mechanism which sorts innervating axons within the target relies on graded differences in the display of target cell factors that selectively promote (or inhibit) axonal growth, combined with a graded susceptibility to these factors among the pool of innervating axons. Perhaps the best illustrated example of this mechanism is the topographically ordered projection of retinal axons onto the optic tectum, in birds and fish, which is regulated by tectal cell-derived ephrin signaling proteins and their cognate receptors differentially expressed in a topographically graded fashion by the retinal ganglion cells. The same molecular mechanism guides motor axons that have cell bodies in neighboring regions of the spinal cord to innervate different domains within multisegmental muscles, such as the diaphragm and intercostal (rib) musculature (Wigston and Sanes, 1982; Laskowski and Sanes, 1987; Feng *et al*., 2000). However, for reasons of mechanical stability, the myofibers that comprise a motor unit are not fasiculated in a contiguous bundle. Rather, they are dispersed throughout the host muscle. This makes it impossible for a single motor axon to acquire a motor unit's worth of muscle fibers, or selectively innervate fibers of a particular type, simply by colonizing a small domain of the target muscle. Instead, axons and muscle fibers must exchange specific information during development that further biases the final outcome to favor certain matches.

A final complication in the apparent simplicity of muscle innervation is that most of the muscle is actually refractory to innervation. NMJs are much larger than most interneuronal synapses (Fig. 5). Nevertheless, they typically occupy only a small fraction of the muscle fiber's total surface area, leaving more than 99% uninnervated. Experimental attempts to form additional synapses in extrasynaptic portions have shown that existing synaptic sites actively suppress the formation and maintenance of novel synaptic sites. Mechanisms regulating the muscle's susceptibility to innervation are discussed in a later section. One consistent finding is that synaptic transmission and evoked activity in the muscle are important factors.

In summary, the apparent simplicity of neuromuscular innervation in mature muscles belies an underlying organizational complexity. Mature patterns of innervation arise through interactions between motor nerves and their target muscles that regulate the cumulative assembly and disassembly of individual synapses. It is worth noting that these conclusions resonate with initial studies of synaptogenesis in other systems, including the mammalian brain. We next review the structure of an individual neuromuscular synapse, before considering mechanisms that direct its development.

Synaptic Specializations at the Neuromuscular Junction

The NMJ is composed of three cell types: Motor neuron, skeletal muscle fiber, and Schwann cell (Fig. 9). The synaptic portion of each of these cells is morphologically and biochemically specialized to support neurotransmission. The extracellular matrix that fills the synaptic cleft is also specialized compared to the matrix that covers the extrasynaptic surfaces of nerve and muscle fibers.

Morphological Specialization

As previously noted, the skeletal muscle endplate occupies a very small region of the muscle fiber surface, usually located midway along the fiber's length. A central location allows action potentials generated at the synapse to spread most rapidly to the ends of the fiber, speeding and synchronizing contractions. The mature endplate is characterized by three morphological hallmarks. First, the postsynaptic surface is impressed with shallow channels and pits, sometimes called synaptic gutters. These postsynaptic depressions hold the branches of the nerve terminal and constitute the *primary synaptic cleft*. Second, the surface of the synaptic gutter is interrupted by a series of invaginations, prosaically named *folds*, which extend several microns into the subsynaptic sarcoplasm and thus constitute a set of *secondary synaptic clefts*. Folds are unique features of the neuromuscular synapse. One possible benefit of forming secondary clefts is to increase the fidelity of synaptic transmission at high firing frequencies by speeding the clearance of spent neurotransmitter from the primary synaptic cleft. Third, the postsynaptic membrane is thickened by an extremely high concentration of AChRs and a coterie of receptor-associated proteins. Synaptic AChRs are concentrated 1,000-fold above levels in extrasynaptic regions of the muscle membrane. Moreover, synaptic AChRs are asymmetrically distributed between the primary and secondary postsynaptic membranes. AChRs are concentrated in the primary postsynaptic membrane, at the crests of the folds. In contrast, the secondary postsynaptic membrane (in the depths of the folds) contains high concentrations of voltage-gated sodium (Na_v) channels.

The motor nerve terminal is also characterized by three morphological hallmarks. First, the motor axon ends in a series of branches, known as a terminal arbor. Unlike the long slender axon, the terminal branches are relatively short and swollen ("varicose"). Second, terminal varicosities are loaded with synaptic vesicles and mitochondria. Third, the synaptic membrane of the nerve terminal contains a large population of active zones, which appear in the electron microscope as thickened regions of the presynaptic membrane associated with several synaptic vesicles.

Within the nerve terminal, the distributions of synaptic vesicles and mitochondria are polarized. The mitochondria,

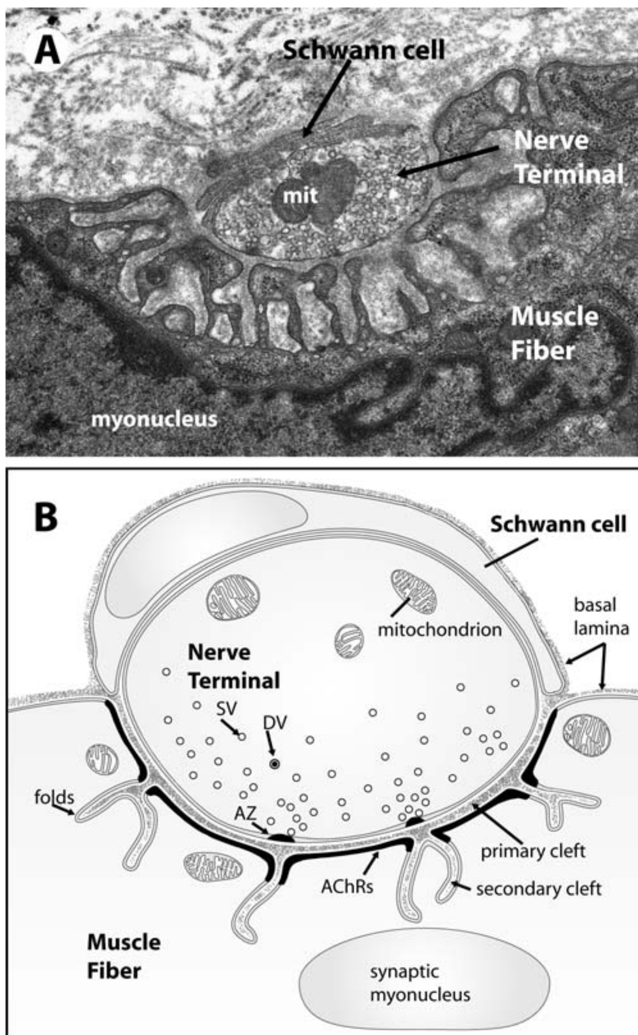


FIGURE 9. Organization of synaptic specializations at the neuromuscular junction (NMJ). Neuromuscular synapses comprise primary and secondary specializations in three cells. Primary specializations include a terminal Schwann cell that caps rather than wraps the motor nerve; a varicose nerve terminal, which accumulates mitochondria (mit) and synaptic vesicles (SV); and a high concentration of ACh receptors (AChRs) and scaffolding proteins in the postsynaptic membrane, which therefore appears thickened in electron micrographs. Secondary specializations develop postnatally and enhance neurotransmission. In the nerve terminal, active zones (AZ) appear along the junctional surface, and the distributions of synaptic vesicles and intra-terminal organelles become polarized with respect to the synaptic cleft. In the muscle, secondary synaptic clefts create folds in the postsynaptic membrane; postsynaptic membrane proteins are distributed asymmetrically in postsynaptic membranes, with AChRs concentrated in the primary postsynaptic membrane, and voltage-gated sodium channels in secondary postsynaptic membranes. Myofiber nuclei in the subsynaptic sarcoplasm express genes for postsynaptic proteins, such as AChR subunits, at higher levels than extrasynaptic myonuclei. A basal lamina (BL) covers the surfaces of muscle fiber and Schwann cell, and fills the primary and secondary synaptic clefts. Although separated by the synaptic BL, active zones in the nerve terminal surface are accurately aligned with postsynaptic folds. Thus, each cell adopts synapse specific behaviors that reflect specializations in its partners. (A) Electron micrograph of an adult mouse neuromuscular junction. (B) Interpreted view of cellular specializations at the vertebrate neuromuscular junction. DV, dense core vesicle.

microtubules, neurofilaments, and other components shared with the axon are concentrated in the center or abjunctional parts of the terminal, away from the synaptic cleft. In contrast, the synaptic vesicles are concentrated in the portion of the terminal nearest the synaptic cleft, and even further concentrated near active zones. The active zones themselves are spaced at intervals along the synaptic surface of the nerve terminal. The most striking example of active zone organization may be at frog NMJs, where they form a set of parallel stripes evenly spaced at 1 μm intervals across the straight terminal branches.

The geometry of the endplate and nerve terminal match precisely (Figs. 8–10). Nerve terminal branches conform exactly to the width and length of the endplate's gutters. Postsynaptic membranes concentrate AChRs directly opposite the nerve terminal branches. In addition, the polarized distribution of AChRs and Na_v channels to the primary and secondary postsynaptic membranes, respectively, reflects the polarized distribution of synaptic vesicles in the nerve terminal. Note that those components closest to the primary cleft are directly involved in transmission; those in arrears augment transmission capacity. Perhaps most remarkable, the locations of the active zone complexes in the nerve terminal membrane are maintained in precise register with the mouths of the secondary synaptic clefts that pattern the postsynaptic surface (Fig. 9). Extraordinary size allows the subsynaptic organization of the NMJ to be recognized and imaged. However, apposition of pre- and postsynaptic specializations occurs to some extent at all synapses and is similarly precise at fast chemical synapses throughout the nervous system.

A wealth of data from physiological, molecular, genetic, and imaging experiments supports the notion that the morphological specializations of the NMJ foster high capacity neurotransmission. The large presynaptic area enables the motor axon to release enough ACh neurotransmitter to produce a postsynaptic current that overcomes the muscle fiber's low input resistance. The branching of motor nerve terminal also serves to spread neurotransmission over enough of the muscle fiber surface so that the resulting depolarization in the surrounding muscle membrane reliably generates a myofiber action potential. Active zones ensure that individual neurosecretory events are distributed, speeded, and synchronized throughout the enlarged synaptic area. Such outcomes are fostered by pre-docking synaptic vesicles preferentially at active zones and extended by concentrating and polarizing the distribution of synaptic vesicles and mitochondria within the terminal cytosol. The formation of a postsynaptic gutter increases the synaptic contact area between nerve and muscle, while postsynaptic folds lacking receptors speed the clearance of transmitter from the primary cleft following muscle fiber activation. It may be interesting to consider whether the structural specializations that support synaptic transmission at this (or any other) synapse could have taken another, significantly different form. Could coordinated control of movement have been achieved through alternative signaling mechanisms?

The importance of terminal morphology to synaptic function is illustrated by its highly stereotyped organization across vertebrate species and in different types of muscles. For example, the precise location of presynaptic active zones opposite postsynaptic

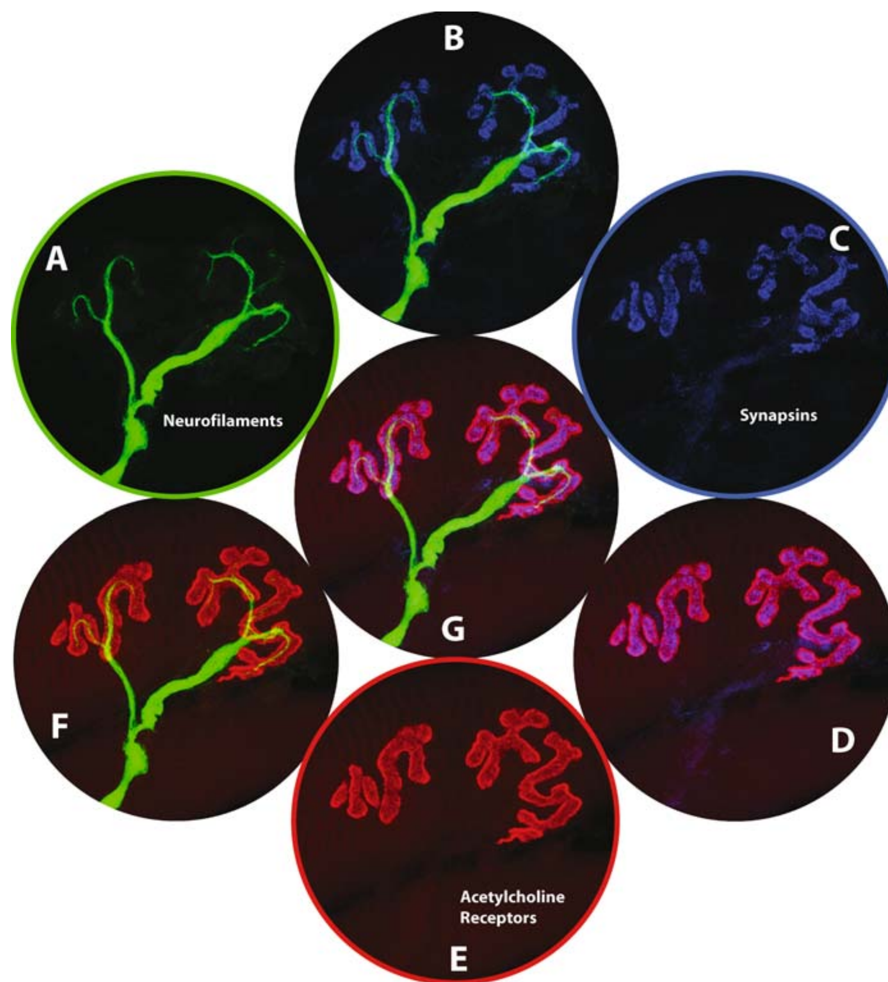


FIGURE 10. Molecular differentiation between nerve terminal and axon. Synaptic structure and function ultimately depend on molecular specializations. In one example, neurofilaments that fill the motor axon are restricted to the core regions of primary branches in nerve terminals (A, and green in other panels). In contrast, synapsins are concentrated throughout the nerve terminal branches and largely absent from the axon (C, and blue in other panels). In the muscle, ACh receptors in postsynaptic membranes (E, and red in other panels) are concentrated 1000-fold above levels in extrasynaptic portions of the myofiber surface. The molecular differentiation between axon and nerve terminal accurately reflects the location of postsynaptic membrane.

folds, described above, occurs in virtually all species examined (from snakes to *sapiens*). Similarly, the distribution of AChRs in the postsynaptic membrane precisely matches the arborization of the nerve terminals, regardless of the shape those terminals may take in a particular muscle or species. Interestingly, terminal arbors and their matching AChR-rich endplate have stereotypical shapes that vary among different muscle types. For example, terminals in jumping muscles in frogs form a series of parallel branches that extend several hundred microns up and down the muscle fiber. In contrast, axons innervating snake muscles that undergo slow and sustained contractions typically form *en grappe* terminals; these contain a cluster of spherical boutons that look like a bunch of grapes and cover only a few tens of microns of the myofiber surface. Similarly in birds and mammals, terminals on fast-twitch fibers form a set of curled branches, like a misshapen pretzel (termed *en plaque*), while terminals on slow-twitch muscles form *en grappe* bouton clusters. In general, long-branched synapses are present where synaptic transmission is strongest and contraction is most vigorous. Smaller, bouton-like terminals are present on muscle fibers that contract more weakly but more sustainably. These distinctive synaptic morphologies have been maintained through hundreds of millions of years of evolutionary divergence, arguing that they impart significant functional advantages. One possible explanation is that an action potential spreads throughout the arbor of an *en plaque* nerve terminal, but spreads unevenly into a subset of the boutons of an *en grappe* nerve terminal. Active zones throughout the *en plaque* terminal branches would be synchronously recruited to neurotransmission, increasing synaptic strength and avoiding failures at periods of maximum muscle contraction. In contrast, active zones in subsets of *en grappe* boutons could be recruited in response to successive action potentials, potentially reducing the synapse's susceptibility to fatigue during extended periods of activity.

Molecular Specialization

A maxim of engineering is that form follows function. The demands of synaptic transmission at the NMJ are supported by structural specializations in pre- and postsynaptic elements, and these in turn are accompanied by biochemical specializations. Some molecular specializations at the synapse, like the concentration of AChRs in the postsynaptic membrane, are so closely allied to synaptic function that they serve as inviolate markers of synaptic sites in the muscle. Other constituents of the synapse have more subtle roles; although they may be concentrated at synaptic sites in all vertebrate phyla, their absence (as, e.g., in genetically engineered mutant mice) causes little perceptible defect in synaptic structure. Nevertheless, their evolutionary conservation suggests that they play essential functional roles in wild animal populations.

The nerve terminal lacks proteins, such as neurofilaments that are concentrated in the axon, and is enriched instead with proteins devoted to the control of synaptic vesicle dynamics (Fig. 10). These include vesicle membrane-associated proteins from the rab, rabphilin, and synapsin families, which regulate intra-terminal synaptic vesicle trafficking; membrane and cytosolic proteins which subserve the docking and fusion of synaptic vesicles with the terminal surface, including synaptobrevin/VAMP, synaptotagmin, SNAP-25, syntaxin, and munc18; voltage-gated calcium channels, which transduce terminal depolarization into the biochemical signal for transmitter release; additional proteins that promote the recovery of vesicle membrane from the terminal surface membrane (clathrin, AP2, dynamin, intersectin), and target the retrieved membrane packets to endosomal compartments (rab 5), where a final complement of enzymes and pumps reconstitute ACh-loaded synaptic vesicles (choline acetyltransferase—ChAT, vesicular acetylcholine transporter—VAChT).

Postsynaptic specializations in the muscle include proteins that establish and maintain the high concentration of AChRs directly opposite the nerve (Fig. 11). Central players in the initiation of AChR clustering opposite the nerve include MuSK (Muscle Specific Kinase), a receptor tyrosine kinase that initiates intracellular signaling in response to agrin, and rapsyn, a receptor-associated scaffolding protein. Their roles are elaborated in a further section. Also concentrated in the postsynaptic membrane is a second large transmembrane protein complex known as the DGC (dystroglycan-associated glycoprotein complex). The DGC is a multifunctional receptor composed of dystroglycan (α and β), the sarcoglycans (α, β, and γ), and sarcospan. It serves as the primary membrane-spanning link between the extracellular matrix in the synaptic cleft and the intracellular cytoskeleton. Primary extra-cellular ligands include the glycoprotein laminin-4 (the α2β2γ1 isoform of the laminin heterotrimer), and the heparan sulfate proteoglycans, agrin and perlecan. Each of these synaptic basal lamina components contains an LG-domain that binds to the α-dystroglycan component of the DGC. (LG-domains are protein modules first identified in *l*aminins and possessing a *g*lobular tertiary structure.) Perlecan appears to be the primary binding partner for AChE in the synaptic cleft, such that interaction between the DGC and perlecan appears to be especially important in maintaining the high concentration of AChE in the synaptic cleft.

DGC interactions with the cytoskeleton include the submembranous cytoskeletal proteins dystrophin and utrophin, which have similar structures and overlapping functions.

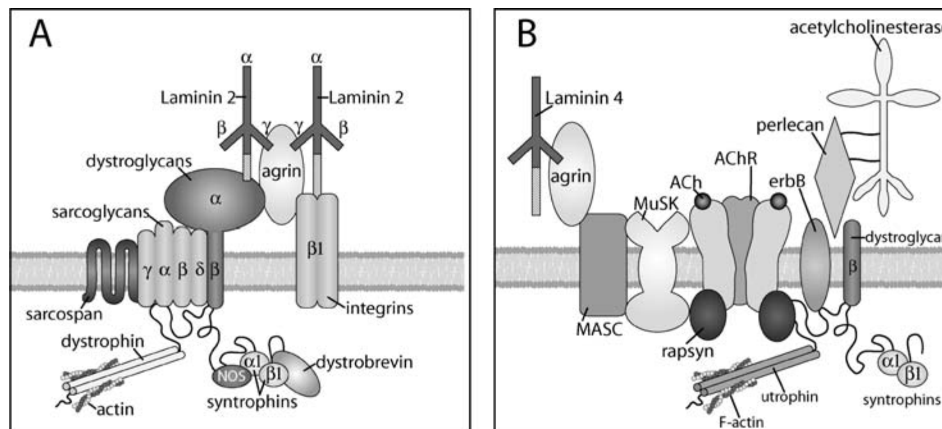


FIGURE 11. Postsynaptic scaffolds at the neuromuscular junction. The dystroglycan–glycoprotein complex in synaptic and extrasynaptic portions of the muscle. (A) In extrasynaptic portions of muscle fibers, the extracellular matrix is comprised of collagens, laminins, and Z(0) forms of agrin. These molecules interact with the cell surface by interactions with α-dystroglycan and β1-integrins. Within the membrane, β-dystroglycan and the sarcoglycans form a complex that binds to dystrophin intracellularly. Dystrophin then interacts with the actin cytoskeleton and with signaling molecules such as dystrobrevins, syntrophins, and nitric oxide synthase. (B) At the synapse, distinct isoforms of laminin and Z(+) agrin interact with different receptors at the cell surface. Agrin activates the tyrosine kinase receptor MuSK via a hypothetical accessory protein MASC. This interaction results in the clustering of pentameric acetylcholine receptors. Intracellularly, the scaffolding protein rapsyn aggregates AChRs and also binds to utrophin. Like dystrophin, utrophin links this complex to the actin cytoskeleton and to syntrophins for signal transduction. In addition to AChRs, erbB receptors and β-dystroglycan are also linked to this complex via utrophin, thus allowing the transduction of signals such as neuregulin in addition to acetylcholine synaptic transmission.

One function of the dystrophin-rich cytoskeleton is to anchor the specialized protein complexes of the postsynaptic membrane to the intracellular cytoskeleton (Fig. 11A). This function has in fact been best studied in extrasynaptic regions of the muscle, where interactions between dystrophin and the DGC stabilize the myofiber membrane during muscle contractions. In extrasynaptic muscle, dystrophin and the DGC are concentrated at costameres. Costameres are membrane-bound protein complexes that tether the sarcolemma to the contractile apparatus of the sarcomere. Mutations to dystrophin or to components of the DGC destabilize this linkage and cause severe forms of muscular dystrophy (Durbeej and Campbell, 2002). A similar set of interactions with postsynaptic membranes likely stabilizes the endplate during muscle activity.

In addition, at synaptic sites, the DGC serves as the membrane platform for utrophin-dependent interactions with the AChR-complex, and with a family of proteins called syntrophins (Fig. 11B). Utrophin specifically associates with MuSK and is concentrated in the AChR-rich primary postsynaptic membrane, along the crests of the junctional folds. (Dystrophin is concentrated in complementary fashion along the secondary postsynaptic membrane deep in the junctional folds, where Na_v channels are concentrated.) Syntrophins are co-concentrated with utrophin at synaptic sites. Little utrophin accumulates in the postsynaptic membrane in the absence of syntrophin, in mice bearing targeted syntrophin gene deletions. The mature structure of the endplate is adversely affected, as few postsynaptic folds are formed in syntrophin-deficient mice (Adams *et al*., 2000). An additional, abbreviated homologue of dystrophin called dystrobrevin is also concentrated at postsynaptic sites. Interestingly, postsynaptic sites in dystrobrevin-deficient mice are initially well formed, but begin to fragment during postnatal development (Grady *et al*., 2000). These and other results suggest that the DGC and its associated cytoskeletal partners promote the growth and maturation of the muscle's postsynaptic specialization (Cote *et al*., 1999; Albrecht and Froehner, 2002).

Postsynaptic differentiation also includes changes in gene expression by synaptic myonuclei. Each muscle fiber is a syncytium, containing hundreds of myonuclei from the fusion of myoblasts during embryonic development. Most myonuclei are spread more or less evenly through the fiber, but several nuclei are prominently clustered beneath the synaptic endplate. Muscle-specific proteins, such as skeletal muscle myosins and muscle creatine kinase, are highly expressed by myonuclei throughout the muscle. In contrast, several synapse-specific proteins are only expressed at high levels by synaptic myonuclei. These include the genes for subunits of the AChR and Na_v channels, for acetylcholine esterase (AChE) and for utrophin. High-level expression of synaptic components may be necessary to support the large size of the NMJ. Conversely, local synthesis may also be a mechanism to ensure that synaptic components are properly targeted to the synaptic sites along the muscle fiber. At interneuronal synapses, as well, there is growing evidence that local synthesis of synaptic components contributes to synapse formation and plasticity (reviewed by Steward and Schuman, 2001). In these cases, however, where the postsynaptic cell has one nucleus, it is specific RNAs rather than specialized nuclei that are sequestered at synaptic sites.

Schwann Cells

Most synapses are surrounded by glial cell processes. At the NMJ, the motor nerve terminal is covered by the processes of one or a few Schwann cells. Although Schwann cells do not have a primary role in synaptic transmission, they play critical roles in supporting nerve terminal metabolism and influence overall levels of innervation in muscle. Like motor nerves and muscle fibers, Schwann cells at synaptic sites are distinguished from their extrasynaptic counterparts by structural and molecular differences.

The most obvious difference is the behavior of the Schwann cell's processes. Schwann cells located in the nerve myelinate motor axons by wrapping sheet-like processes around axon segments, in compact, concentric circles. Schwann cells at synapses do not wrap axon terminals. Rather, they extend short processes that cover the non-synaptic surface of the terminal (Fig. 9). Synaptic Schwann cell processes tend to follow the course of the terminal branches; they avoid the synaptic cleft and rarely extend into extrasynaptic muscle.

Molecular differences accompany the morphological differences between preterminal and terminal Schwann cells. For example, myelinating Schwann cells express specific transcription factors, including krox-20 and Oct-6/SCIP/Tst-1, and their membranes contain a unique complement of membrane glycolipids and glycoproteins that includes protein-zero, peripheral myelin protein 22, periaxin, and myelin-associated glycoprotein. In contrast, synaptic Schwann cells express little or no myelin-associated proteins, but do express high levels of krox-24 (also called EGR-1, zif/268, and NGFI-A), the intracellular calcium-binding protein S100, the cell-surface adhesion molecule NCAM, and the extracellular matrix component laminin $\alpha 4$. It is unlikely that these differences represent fully differentiated cell fates, for although synaptic and myelinating Schwann cells derive from common neural crest progenitors, their final state depends on which portion of which axon they contact and is reversible (Garbay *et al*., 2000; Lobsiger *et al*., 2002). Signals associated with large caliber motor and sensory axons cause their associated Schwann cells to form myelin. Following axonal degeneration, the myelinating Schwann cells revert to a pre-myelinating phenotype, a process that includes the downregulation of myelin proteins and upregulation of S100, NCAM, and laminin $\alpha 4$. One possibility is that synaptic Schwann cells are permanently pre-myelinating. Alternatively, unknown synaptic signaling factors may induce a uniquely differentiated Schwann cell state. In any event, factors concentrated in the synaptic cleft prevent Schwann cells from myelinating the terminal portion of the axon. One factor contributing to exclusion of Schwann cell processes from the synaptic cleft is laminin-11, a component of the synaptic cleft material that inhibits the motility of Schwann cell processes (Patton *et al*., 1998).

The Synaptic Cleft

At most chemical synapses, the gap between the pre- and postsynaptic elements is filled with a matrix of glycoproteins and proteoglycans. At interneuronal synapses, where the cleft is only 20 nm across, the synaptic matrix most likely consists of the extracellular domains of membrane proteins. In the synaptic cleft at the NMJ, the synaptic matrix contains secreted glycoproteins and proteoglycans assembled into a tightly woven sheet of material known as a basal lamina. The thickness of this structured matrix largely determines the 50–70 nm width of the neuromuscular synaptic cleft. As we shall see, the structure and activity of the synaptic basal lamina plays an essential role in the formation and maintenance of the NMJ, as well as its physiology (Patton, 2003).

Basal laminae are present in many tissues in the body, serving as substrates for cell adhesion and movement, imparting structural integrity, and organizing cell-signaling domains through membrane receptors such as integrins. In muscle, the synaptic matrix is part of a continuous basal lamina that covers the entire surface of each myofiber, and which has been structurally likened to a nylon stocking on a leg. A similar basal lamina made by Schwann cells covers each peripheral nerve fiber. Extrasynaptically, the myofiber basal lamina is closely bound to the surface of the myofiber; in the synaptic cleft, the basal lamina is bound directly to the surfaces of both nerve terminal and endplate. Although structurally similar, synaptic and extrasynaptic portions of the myofiber basal lamina contain distinct molecular components. In particular, the synaptic cleft contains distinct isoforms of the main structural components of the basal lamina, and in addition, incorporates a number of unique accessory factors.

The principal components of all basal laminae are laminin, type IV collagen, entactin (also called nidogen), and the heparan sulfate proteoglycan, perlecan (Fig. 12). Laminins and collagens IV are families of long, rope-like glycoproteins. Their structure comes from a trimeric composition of homologous subunits (here referred to as "chains") that are entwined along much of their length. Laminins and the type IV collagens self-polymerize, forming supramolecular networks cross-linked by entactin. Together, they account for much of the structural integrity of the basal lamina. Interestingly, synaptic and extrasynaptic basal laminae contain different isoforms of these components, which differ in chain composition. The extrasynaptic basal lamina primarily contains collagen IV trimers composed of α1(IV) and α2(IV) chains, and laminin-2 (the α2β1γ1 heterotrimer). Synaptic basal lamina is more complicated. It contains collagen IV composed of the α3(IV), α4(IV), and α5(IV) chains and also contains three distinct laminin heterotrimers: Laminin-4 (α2β2γ1), laminin-9 (α4β2γ1), and laminin-11 (α5β2γ1). The synaptic matrix also contains an uncharacterized variant of entactin, possibly nidogen-2 (Chiu and Ko, 1994).

Synaptic laminins principally differ from extrasynaptic laminins by virtue of their β2-chain and differ from each other by their α-chain component, discussed in more detail below. The laminin-β2 chain was originally named s-laminin, by Hunter, Merlie, and Sanes, for its *s*ynaptic concentration (Sanes *et al*., 1990). Most synaptic basal lamina components are present throughout the synaptic cleft. One exception is laminin-9, containing the α4 chain, which is absent from postsynaptic folds, and is concentrated in small patches within the primary cleft (Patton *et al*., 2001). The several synaptic laminins have

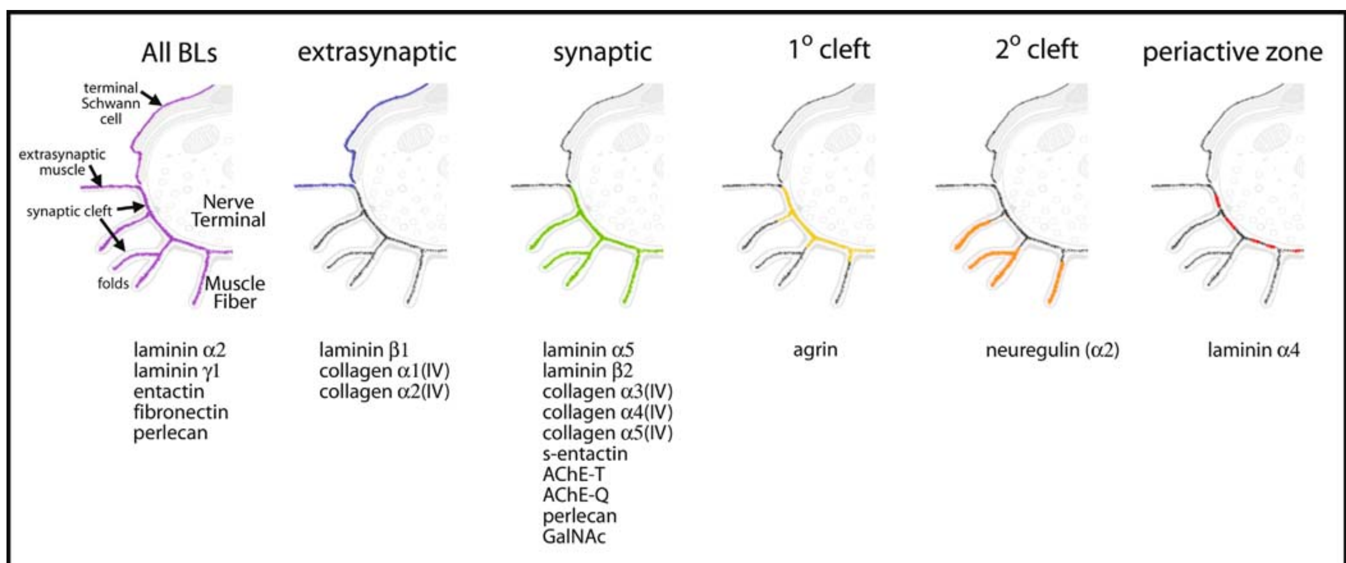


FIGURE 12. Specialization of the synaptic basal lamina. The basal lamina (BL) that ensheaths each myofiber is molecularly specialized at synapses. All BLs contain type IV collagen, laminin, and entactin, which together provide structural integrity. Synaptic and extrasynaptic BLs differ in specific isoforms of these ubiquitous components. In addition, synaptic BLs are enriched by a series of accessory components. These include the catalytic and collagen-like tail subunits of acetylcholine esterase (AChE-T and AChE-Q, respectively), the heparan sulfate proteoglycans agrin and perlecan, and the growth and differentiation factor neuregulin. Agrin, neuregulin, and the laminin α4 subunit have restricted distributions within the synaptic BL. (Figure reprinted from Patton, 2003, with permission.)

important roles in the maturation of the nerve terminal and the terminal Schwann cell, which are discussed later.

The synaptic basal lamina also incorporates a number of accessory constituents, including agrin, neuregulin, a glycosyltransferase, and a collagen-tailed form of AChE. Agrin and neuregulin are signaling molecules that act through membrane receptor tyrosine kinases to control the expression and distribution of synaptic components in the muscle fiber. Agrin is concentrated in the primary synaptic cleft, along the crests of the AChR-rich junctional folds (Trinidad *et al*., 2000). In contrast, at least one isoform of neuregulin is concentrated in the troughs of the postsynaptic folds and is absent from the primary cleft. The roles of agrin and neuregulin in postsynaptic differentiation are addressed below.

Although our understanding of the role of glycosylation at the synapse is rudimentary, several components of the synaptic cleft bear unique and evolutionarily conserved glycosylation patterns. For example, VVA-b4 isolectin (isolated from the hairy vetch, *vicia villosa*) recognizes a carbohydrate group (terminally sialylated *N*-acetyl galactosamine) that is specifically concentrated at NMJs in nearly all classes of vertebrates: fish, amphibians, reptiles, birds, and mammals. This suggests that a role for specific glycosylation patterns developed early in vertebrate evolution and was important enough to be preserved for hundreds of millions of years in all viable offspring. One possibility suggested by recent studies is that glycosylation of the dystroglycan receptor alters its affinity for variants of laminin, agrin, and perlecan, fostering their concentration in the synaptic cleft (Xia *et al*., 2002).

Perlecan plays a specific and important role in binding AChE into the synaptic cleft (Peng *et al*., 1999). Perlecan is a long, multidomain molecule, with binding sites for a number of extracellular matrix proteins and receptors. One likely partner is ColQ, a collagen-like subunit of AChE present in the synaptic cleft. Mutations in the genes for perlecan and ColQ each prevent the accumulation of AChE at synaptic sites (Donger *et al*., 1998, Feng *et al*., 1999, Arikawa-Hirasawa *et al*., 2002).

AChE hydrolyzes acetylcholine following synaptic transmission at the NMJ. Because nicotinic AChR channels in muscle do not rapidly inactivate, AChE effectively terminates neurotransmission by eliminating ACh as it dissociates from the AChRs. Two specializations support this role. First, AChE's rate of catalysis is extraordinarily fast. The measured k_{cat} is 14,000 s^{-1}, and the k_{cat}/K_M value is calculated to be $1.6 \times 10^8\ (Ms)^{-1}$, which is near the diffusion limited rate for enzymatic reactions. AChE is essentially catalytically perfect. Second, AChE is concentrated to extraordinarily high levels in the synaptic basal lamina, up to 3,000 per μm^2 (Rogers *et al*., 1969; Salpeter, 1969; Salpeter *et al*., 1978; Anglister *et al*., 1998). The dependence of synaptic function on AChE activity is shown by the potency with which anticholinesterase drugs affect neuromuscular synaptic transmission. Anticholinesterases are administered at low doses to myasthenic patients, to bolster weak neuromuscular transmission. At moderately higher doses, however, anticholinesterase exposure is lethal. AChE is a primary physiological target of organophosphate and carbamate insecticides used on crops and livestock, and military nerve gases stored for use against humans.

Collectively, the components of the synaptic cleft reflect the organization of the pre- and postsynaptic cells. While most components of the synaptic basal lamina are synthesized by the target muscle fiber, including the laminins, collagens, perlecan, and cholinesterase, the nerve supplies components as well, most notably agrin. In principle, specialization of the synaptic cleft could act primarily during maturation of the maturation factors, to strengthen transmission and mechanically stabilize the synaptic site during activity. In this case, the first evidence of specialization in the synaptic cleft might appear well after the establishment of initial synaptic contacts. Direct observations show, however, that most components of the synaptic cleft are present at early stages of synapse formation, and several, such as AChE and laminin-β2, are highly concentrated at synaptic sites shortly after their formation. This raises the alternative possibility that the restricted distributions of synaptic cleft components may reflect a direct role in organizing nerve terminals and endplates. Indeed, agrin, laminin β2, and neuregulin were originally identified by attempts to define the molecular signals that promote synaptic differentiation. These possibilities are explored more fully in the next section, where the molecular mechanisms underlying synaptic development and maturation are considered.

SYNAPSE FORMATION

From our discussion of synaptic structure and function, we conclude that there are two essential aspects to the formation of a chemical synapse. Small regions of the axon and its target cell become specialized to support effective neurotransmission, and these synaptic specializations are directly apposed to each other across a synaptic cleft. In broad terms, it is possible to imagine two alternative processes by which synaptic specializations in the motor axon and muscle fiber are formed and colocalized. In one case, cell autonomous programs of development in the nerve and muscle first produce synaptic specializations in each cell, independently; these specializations then become oriented with respect to each other through intercellular interactions. Alternatively, synaptic differentiation could be initiated by local signaling interactions between nerve and muscle. In this case, the location of the synaptic site could reflect sites of initial contact, or regions of especial susceptibility along the cell surfaces.

In support of the preprogrammed model of synaptic differentiation, muscle fibers and motor neurons do independently express most of the components of the mature NMJ and will organize primitive synaptic structures when cultured separately. For example, cultured myotubes (immature muscle fibers) express functional AChRs on their surface and spontaneously cluster receptors in small patches, similar to AChR plaques that form *in vivo*. Cultured myotubes also respond to application of neurotransmitter with weak contractions. Similarly, the neurites of motor neurons cultured in the absence of muscle cells are capable of spontaneous and evoked release of neurotransmitter. Nerves and muscles are therefore prepared to form rudimentary synaptic specializations without direction. Synaptic connections

might then be established by either guided or random intersections of pre- and postsynaptic specializations. For the mature pattern of innervation observed in adult muscles to emerge by this mechanism, *bona fide* synapses would require stabilization, while ectopic (unconnected) synaptic specializations would require disassembly. In fact, synapse formation in muscle does include a partial misalignment of initial specializations, an enhancement of well-formed connections, and the elimination of weakly matched specializations. These events are discussed in further detail, below. In effect, mature synapses are sculpted from an initial population of specializations that are more crudely aligned. This model recalls earlier stages of neural development in vertebrates, wherein the final population of neurons and axonal projections represent a subset of those initially formed.

In the alternative scenario, synapsing cells directly organize each other's synaptic specializations, through diffusible and/or cell-surface cues. In fact, several direct observations support this signaling model of synapse formation at the NMJ. First, in studies of nerve–muscle cocultures, developing myotubes assembled a new AChR-rich postsynaptic apparatus at sites where they were contacted by axons (Fig. 13) (Anderson and Cohen, 1977; Anderson *et al*., 1977). Preexisting postsynaptic specializations, which had formed spontaneously (without innervation), were not preferentially innervated by axons and were often *disassembled* in response to novel innervation of the myotube (Kuromi and Kidokoro, 1984). Second, presynaptic specializations preferentially formed where axons contact muscle surfaces, in nerve–muscle cocultures (Fig. 14), during normal

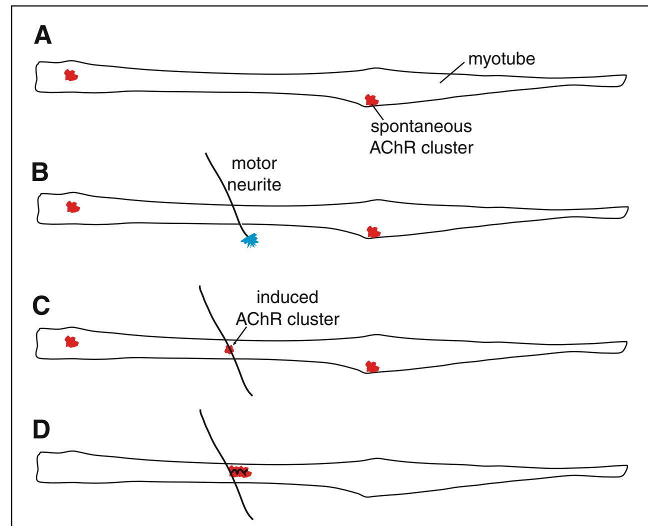


FIGURE 13. Motor nerves organize postsynaptic specializations in cultured muscle cells. Experiments in which spinal motor neurons were cocultured with differentiated muscle myotubes and then stained with α-bungarotoxin to show the distribution of ACh receptors (AChR), showed that (A) muscle cells are capable of clustering AChRs "spontaneously," independent of innervation; (B) motor neurites do not target spontaneous AChR clusters for preferential innervation; (C) neurites induce new AChR clusters where they contact the muscle fiber; and (D) maturation of nerve-induced AChR clusters is accompanied by disassembly of spontaneous clusters. These results, obtained in studies by Cohen and Anderson, and Frank and Fischbach, suggested that motor nerves provide signals that organize the local differentiation of the postsynaptic site.

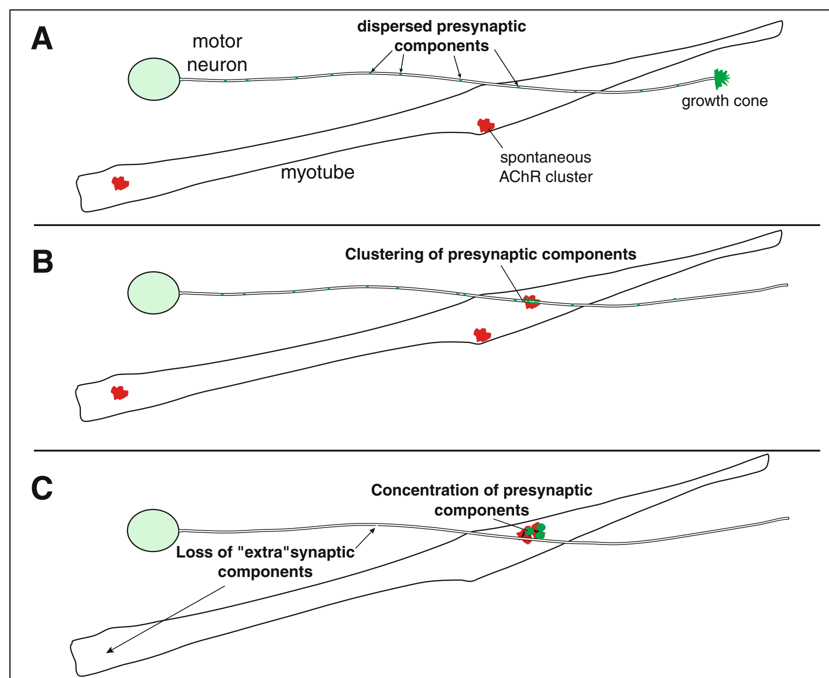


FIGURE 14. Myotubes promote presynaptic differentiation in cultured motor axons. In nerve–muscle coculture experiments by Lupa and Hall, developing motor neurites preferentially concentrated synaptic vesicles at sites of contact with muscle cells. Presynaptic sites coincided spatially and temporally with postsynaptic sites induced by the neurite in the muscle cell. The results indicated that muscle fibers provide local, retrograde signals that promote presynaptic differentiation in the axon.

development, and during reinnervation of muscles following nerve injury *in vivo* (Cajal, 1928; Marshall *et al*., 1977; Lupa and Hall, 1989; Lupa *et al*., 1990). Third, nerve terminals and muscle endplates develop in concert as synaptogenesis proceeds during normal development, *in vivo*. The initial axonal projection predicts the final location of synapses in the muscle, and early errors in matching terminals and endplates are rapidly corrected. Indeed, it is difficult to imagine how the highly stereotyped geometry of the mature nerve terminal could reliably match the AChR-rich endplate membranes, or how the position of terminal active zones could so accurately align with postsynaptic folds, through completely independent programs of development in nerve and muscle.

Historically, these observations provided strong *a priori* evidence that the organization of synaptic specializations is a concerted process regulated by locally produced nerve- and muscle-derived factors. Considerable molecular evidence has been added over the last 25 years. Nerve-derived factors that organize the muscle endplate have been identified and muscle-derived factors have been identified as candidate cues to guide nerve terminal formation and regulate Schwann cell motility. As a result, the signaling model of synapse formation has come to dominate our thinking of how most chemical synapses are established and maintained.

Recently, the signaling model has been challenged to accommodate earlier observations that nerve terminals and AChR-rich postsynaptic specializations are often misaligned during the earliest stages of synapse formation in muscle (Braithwaite and Harris, 1979; Lupa and Hall, 1989; Dahm and Landmesser, 1991). Most dramatically, it has recently been discovered that nearly normal patterns of postsynaptic differentiation in the muscle occur in the complete absence of a nerve (Yang *et al*., 2000, 2001; Lin *et al*., 2001). Discussed more fully in a later section, the implication of this surprising result is that innervation is strongly influenced by a preestablished pattern of synaptic differentiation in the muscle, but is initially imprecise. Synaptic specializations in the nerve and muscle then rapidly align with each other through an exchange of synaptogenic signals. Signals from the nerve may either stabilize the initial synaptic template in the muscle, or disassemble and replace it. Thus, the exact roles of signaling and preestablished programs in synapse formation remain uncertain, even at the NMJ. It seems quite possible that elements of both models of synapse formation, preprogrammed and signal-mediated, may be required to fully explain synapse formation as it occurs during development.

Presynaptic Differentiation

The first motor axons to grow into developing muscles arrive at or before the time myoblasts fuse to form myotubes (Bennett, 1983). Individual motor axons branch many times within the muscle to innervate the many fibers that eventually comprise a motor unit. Axons likely respond directly to guidance cues provided by developing muscles, although guidance to synaptic sites after injury in adults is largely mediated by Schwann cells (Son and Thompson, 1995; Riethmacher *et al*., 1997; Nguyen *et al*., 2002).

Developing motor axons release ACh at their growth cones and are capable of activity-dependent neurotransmission within minutes of contact with the myotube (reviewed in Sanes and Lichtman, 1999). Nevertheless, synaptic coupling at nascent synapses is very weak, due in part to the small number of synaptic vesicles present in the primitive terminal, and the low density of AChRs in the postsynaptic membrane. The first overt sign of presynaptic differentiation is the formation of small branches and varicosities at the end of the axon, which occurs after nerve–muscle contact *in vivo*. Terminal varicosities initially form in the vicinity of concentrated "plaques" of AChRs located on the surface of the muscle fibers. (Whether these AChR plaques are targets of, or are induced by, the innervating nerve is not yet clear.) Within a day or two, nerve terminals and receptor plaques are topographically matched: Each postsynaptic plaque of receptors is covered by a cluster of terminal varicosities, and few terminal branches stray beyond the edges of a receptor plaque. During the following week, nerve terminals concentrate synaptic vesicles at high densities, lose the microtubule structures of the axon, and widen to cover more of the postsynaptic surface. Finally, mitochondria accumulate, the terminal becomes polarized, and active zones form along the synaptic portion of the terminal membrane. Maturation of the terminal is accompanied by a large increase in the number of vesicles released per depolarization and thus a dramatic increase in the strength of the synaptic connection.

One conclusion from these observations is that presynaptic differentiation is largely a process of organization, with progressively higher levels of detail in successive steps. Presynaptic differentiation proceeds from the segregation of terminal and axonal compartments, through increasing levels of structural complexity, with relatively little change in the molecular components of the nerve terminal. A second lesson is that nerve terminal organization progresses gradually, in steps. Primitive synaptic connections form as presynaptic terminals and postsynaptic receptors become colocalized. Immature nerve terminals form as the levels of primary synaptic components increase. Mature nerve terminals appear as the synaptic components are further organized to concentrate neurotransmission at active zones. An emerging theme is that each of these steps may be separately regulated by factors derived from the muscle and/or the terminal Schwann cell.

Presynaptic Differentiation Factors

Nearly a century ago, Cajal surmised that muscles supply factors which cause motor axons to form nerve terminals, and that these factors are concentrated at postsynaptic sites. Fernando Tello, a student of Cajal, observed axons of crushed peripheral nerves as they grew back into the denervated muscle (Cajal, 1928). Tello noted that the reinnervating axons stopped growing and formed terminal-like structures at places that appeared to be the original sites of innervation on the muscle fibers. This result was later confirmed when histological stains for cholinesterase were developed and used to show that postsynaptic sites remain identifiable and at least partly intact during extended periods of denervation (McMahan *et al*., 1978). Regenerating motor axons

do in fact demonstrate remarkable synaptic specificity, faithfully reinnervating the tiny fraction (0.1%) of each muscle fiber's surface that was previously occupied by an original nerve terminal.

The implications of Tello's observations were explored more fully by U.J. McMahan and his colleagues, in a series of experiments now 25 years old (Fig. 15). They first noted that the surface of the muscle fiber, including the synaptic endplate, is entirely covered by a basal lamina. Contact between the nerve and muscle is therefore mediated by the myofiber basal lamina, and reinnervating axons form new synaptic contacts over the original synaptic basal laminae. At the time, the cleft material was known to be molecularly specialized, containing acetylcholinesterase (McMahan *et al*., 1978). They therefore supposed that the synaptic basal lamina harbored additional, unidentified components, which controlled the growth and synaptic differentiation of reinnervating axons. To test this idea, they took advantage of an old observation that crushed muscle fibers retract and degenerate within their basal lamina sheath, which remains largely intact for a time. Then, like Tello before them, they observed the growing ends of cut axons during reinnervation of the muscle, although in this case after the muscle fibers had degenerated. Remarkably, not only did motor axons faithfully reinnervate the empty tubes of basal lamina, but new nerve terminals were located immediately adjacent to the original synaptic basal lamina, which was identified by stains for cholinesterase (Marshall *et al*., 1977; Sanes *et al*., 1978). The nerve terminals even assembled active zones in proper alignment with the stems of the basal lamina that previously lined postsynaptic folds (Glicksman and Sanes, 1983). Most importantly, these experiments revealed that the underlying muscle fiber is not required for axons to accurately reinnervate the original synaptic basal lamina. This result strongly implicated the synaptic basal lamina itself as a reservoir of molecular cues that arrest the growth of the motor axon and organize the formation of the motor nerve terminal.

Several components of the synaptic basal lamina have been proposed to regulate presynaptic differentiation of the motor axon. The best understood are synaptic isoforms of the laminin heterotrimer ($\alpha\beta\gamma$), made by the muscle fiber (Fig. 16). Three synaptic laminin heterotrimers have been identified, as described earlier (laminin-4, -9, and -11). Each contains the β2-chain, but differ from each other in their α-chain (α2, α4, and α5, respectively). Synapses form abnormally in mutant mice lacking the laminin β2-chain (Noakes *et al*., 1995), a genetic modification that prevents muscles from synthesizing any of the synaptic laminin trimers. The normal synaptic differentiation of all three cells is perturbed (Fig. 17). Motor nerve terminal organization stalls at an immature stage, with no polarity and very few active zones; the formation of postsynaptic folds is grossly retarded; and synaptic Schwann cells extend processes into the synaptic cleft, nearly isolating the pre- and postsynaptic elements. Not surprisingly, mice lacking the β2-laminins move poorly and typically die at weaning.

In principle, the loss of any or all of synaptic isoforms could cause the synaptic defects seen in the laminin β2-deficient animals. However, comparisons of synaptic defects in mice lacking the individual laminin α-chains, combined with observations

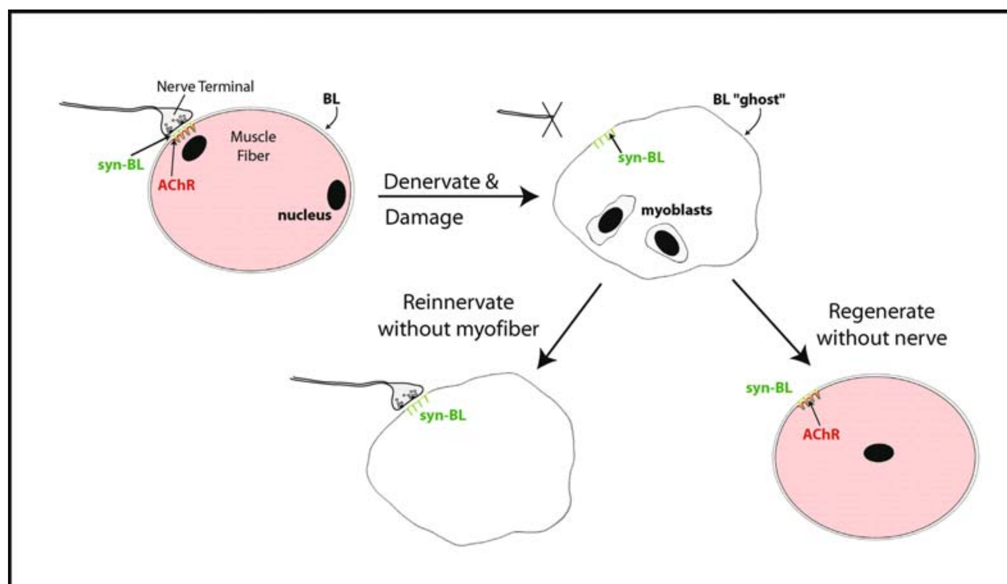


FIGURE 15. Synaptic basal lamina contains synaptogenic cues. Motor axons accurately reinnervate the original synaptic sites on muscle fibers, following axotomy in adult animals. Similarly, new muscle fibers concentrate AChRs at original synaptic sites, following injury-induced degeneration and regeneration. In a series of experiments by McMahon and his group, the ability of motor axons and muscle fibers to accurately target synaptic differentiation to original sites was determined in the absence of synaptic partners. Importantly, the myofiber basal lamina (BL) remains structurally intact during the degeneration and regeneration of a muscle fiber, and synaptic BL could be located by stains for acetylcholine esterase. When muscle fiber regeneration was prevented by irradiation of myoblasts, regenerating axons formed new nerve terminals opposite original synaptic BLs, even in the absence of a muscle fiber. When reinnervation was prevented by ligature, regenerating muscle fibers concentrated AChRs opposite original synaptic BLs, even in the absence of a nerve terminal. The results implied that synaptogenic cues were stably incorporated into the synaptic BL, and that these were sufficient to organize both pre- and postsynaptic differentiation.

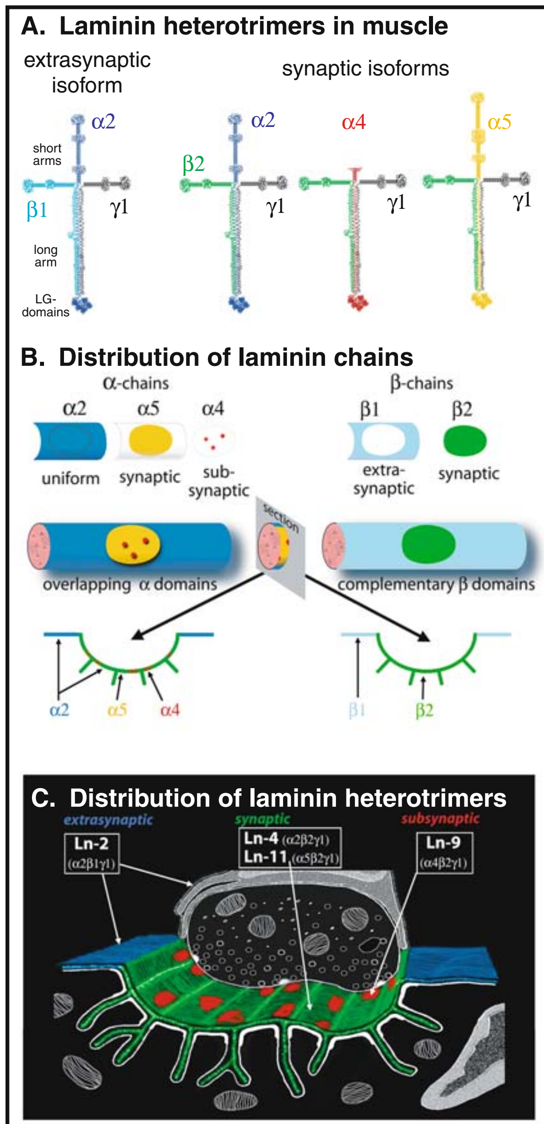


FIGURE 16. Laminin isoforms differ in extrasynaptic and synaptic muscle basal laminae (BLs). Laminins are large (*c.* 800 kDal) heterotrimers of related α, β, and γ chains. (A, B) Muscle fibers express three laminin α-chains: α2 is present in all BLs; α5 is restricted to synaptic BLs; α4 is further restricted to subsynaptic domains within the primary synaptic cleft, adjacent to junctional folds. Two laminin β-chains are expressed in muscle: β1 is restricted to extrasynaptic BL; β2 is concentrated in synaptic BL. All muscle laminins contain the γ1 chain (not shown). (C) Based on the distribution of the α, β, and γ chains: Extrasynaptic BLs contain primarily laminin-2 (α2β1γ1); synaptic BLs contain a mixture of laminins-4, -9, and -11 (α2β2γ1, α4β2γ1, and α5β2γ1, respectively). Specific associations between the distribution of laminin-9 and the location of presynaptic active zones is based on abnormal active zone placement in laminin α4-deficient mice (Fig. 17) and remains speculative. (Reprinted from Patton, 2003, with permission.)

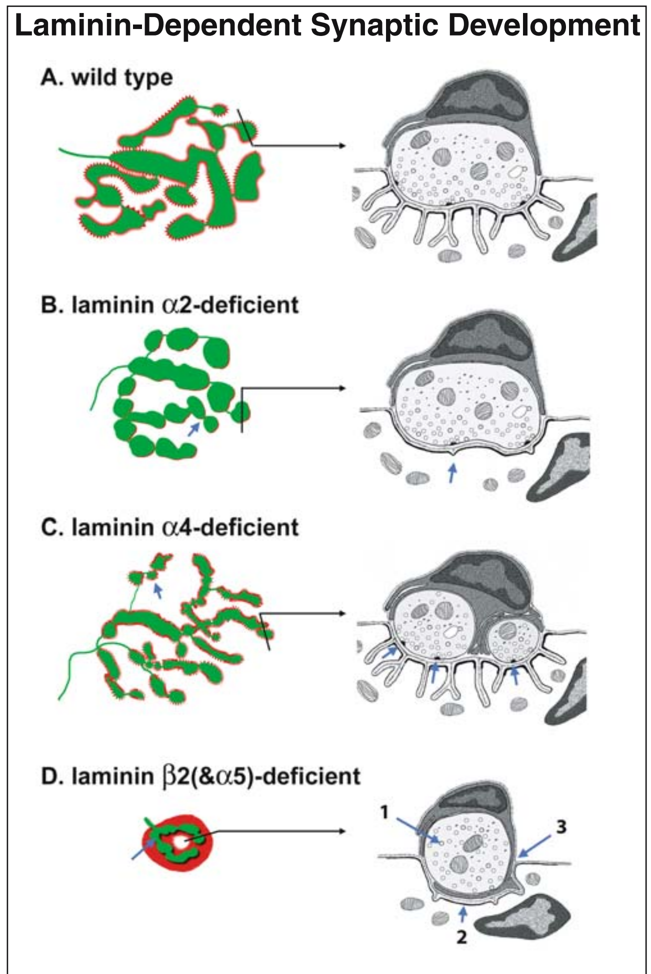


FIGURE 17. Neuromuscular junctions in laminin-deficient mice. Cellular and ultrastructural organization of the neuromuscular junction in normal mice (A) and mice with mutations in specific laminin chains (B–D). (A) In normal muscles, pre- and postsynaptic specializations are precisely aligned. Nerve terminals are embedded in ACh receptor-rich gutters in the muscle surface, synaptic vesicles and ACh receptors are concentrated near the synaptic cleft, and active zones are aligned with secondary synaptic clefts. (B) Loss of laminin α2 prevents the proper formation of postsynaptic folds. Nerve terminal branches are smaller, possibly in compensation for the paucity of folds. (C) Loss of laminin α4 prevents the proper alignment of active zones and secondary clefts, although active zones and folds form at normal frequencies. Terminal varicosities are markedly smaller than in controls. (D) (1) Loss of laminin β2 causes severe synaptic defects. Presynaptic terminals have few branches and lack polarity and active zones; (2) AChR-rich postsynaptic membranes do not match nerve terminal branches and have few folds; (3) Schwann cell processes invade the synaptic cleft, nearly isolating nerve terminal from endplate. The β2-deficient cleft also lacks laminin α5, but contains the laminin β1-chain, which is normally restricted to extrasynaptic muscle. The results indicate that individual synaptic laminins organize postnatal maturation at the neuromuscular junction, including the precise registration of pre- and post-synaptic specializations. (Reprinted from Patton, 2004, with permission.)

of the effects of purified synaptic laminins on the behavior of cultured cells, suggests that each isoform may promote a different aspect of synaptic development.

Laminin-4, which contains the α2-chain, is important for the development of normal postsynaptic folds, but otherwise appears redundant to laminin-9 and -11. Postsynaptic folds form poorly in the absence of laminin-4, in mutant mice lacking the α2-chain, but nerve terminal differentiation and Schwann cell processes appear nearly normal (Fig. 17).

Laminin-9, which contains the α4 chain, is required for the proper alignment of presynaptic active zones and postsynaptic

folds (Patton *et al*., 2001). In normal mice and indeed nearly all vertebrate animals, active zones and folds are precisely aligned across the neuromuscular synaptic cleft. In contrast, mice lacking the laminin α4-chain fail to registrate active zones and folds across the synaptic cleft (Fig. 17). Although lacking in alignment, the active zones and folds form at normal densities in the absence of the α4-laminin chain. Therefore, laminin-9 is not required for presynaptic maturation *per se*. Rather, it acts as a target-derived synaptic organizing factor.

Two unique features of laminin-9 may play a role in guiding the colocalization of active zones and folds. First, laminin-9 is concentrated in small patches in the synaptic basal lamina patches whose distribution reflects the orientation of the postsynaptic folds (Patton *et al*., 2001). Second, presynaptic calcium channels form stable complexes with laminin-9 but not other endogenous laminins, in extracts of Torpedo electric organs (Sunderland *et al*., 2000). Although the directness of their interaction remains uncertain, the concentration of calcium channels at active zones in vertebrates raises the possibility that laminin-9 orients the location of active zones through interactions with presynaptic calcium channels. In support of this idea, targeted disruption of the presynaptic calcium channel in mice causes a similar dislocalization of active zones and folds (Nishimune *et al*., 2002). It could, of course, be the other way round, with active zones directing the location of folds, via laminin-9. In either case, the receptors that mediate selective interactions between synaptic laminins and the postsynaptic membrane are not known.

The remaining synaptic isoform of laminin (laminin-11, containing the α5-chain) might play a more fundamental role in organizing formation of the NMJ. First, synaptic defects in mice lacking laminin-4 and laminin-9 are mild compared to the defects in laminin β2-deficient mice. Second, purified preparations of laminin-11 arrest neurite outgrowth from cultured motor neurons, and recombinant preparations of the β2-chain are capable of inducing the morphological, biochemical, and functional properties of nerve terminals (Patton *et al*., 1997; Son *et al*., 1999). Third, nerve terminal formation is aberrant at embryonic stages in mutant mice specifically lacking laminin-11 through mutation of the α5-chain (Bierman *et al*., 2003).

Laminin-11 appears to play a second important role at the NMJ, one that reveals complex interrelationships between nerve, muscle, and Schwann cell. Normally, Schwann cells cap the nerve terminal. In mutant mice lacking the laminin β2-chain, the terminal Schwann cell invades the synaptic cleft, interrupting neurotransmission (Noakes *et al*., 1995). *In vivo* and *in vitro* experiments implicate laminin-11 as an inhibitory substrate to Schwann cell processes that directly prevents Schwann cell entry into the cleft (Patton *et al*., 1998). Poor nerve terminal differentiation may also contribute to the Schwann cell's misbehavior at laminin β2-deficient synapses.

The synaptic laminins appear to act in concert to organize the behavior of all three synaptic cells. However, their identified roles are largely directed at mid and late stages of synaptic development. What factors regulate the initial transformation of growth cone into nerve terminal?

Candidate factors include agrin, fibroblast growth factors, neurotrophins, and the cell-adhesion molecule NCAM. Most of these have been proposed to regulate aspects of nerve terminal differentiation based on cell culture assays of motor neurons. For example, agrin inhibits motor neurite outgrowth and promotes the clustering of synaptic vesicles, neuronal behaviors that preferentially occur during nerve terminal formation *in vivo* (Campagna *et al*., 1995, 1997). A role for agrin in presynaptic differentiation has been difficult to discern *in vivo*, however. Loss of agrin in mice grossly perturbs postsynaptic differentiation. Moreover, an absence of postsynaptic differentiation by other means, as by loss of the agrin-transducing receptor MuSK, causes a similar absence of nerve terminal formation and increased axonal growth, despite apparently normal levels of agrin.

Similarly, several neurotrophins increase neurosecretion by motor neurons in culture, but their potential role in nerve terminal differentiation *in vivo* is obscured by their strong roles in modulating postsynaptic differentiation. Growth factors, as well, have been implicated in both motor neuron survival and synaptic development. For example, FGF5 accounts for a major fraction of the muscle-derived survival activity when assayed on cultured motor neurons and also increases the expression of choline acetyltransferase by cholinergic neurons *in vitro* (Hughes *et al*., 1993; Lindholm *et al*., 1994). However, mice lacking FGF5 have modest synaptic defects, suggesting that their roles may be limited or may overlap with other unidentified factors (Moscoso *et al*., 1998). Interestingly, FGF2-coated beads cause axonal swelling and synaptic vesicle accumulation at sites of contact along developing motor neurites in culture (Dai and Peng, 1995). Although roles for FGFs have not been established at synapses *in vivo*, their effects on cultured neurons indicate that sustained increases in intracellular calcium may be a critical intracellular determinant of presynaptic differentiation.

NCAM is concentrated in the neuromuscular synaptic cleft *in vivo* and regulates neurite outgrowth in culture. Functional synapses form in NCAM-deficient mice. Thus, NCAM does not play a dominant role in the establishment of this synapse (Moscoso *et al*., 1998). However, additional studies revealed that synapses in adult NCAM-deficient mice retain functional and biochemical features of embryonic synapses (Rafuse *et al*., 2000; Polo-Parada *et al*., 2001). Compared to normal controls, neurotransmission at synapses in mutant muscles was markedly depressed and prone to fail entirely in response to repetitive stimulation. In pursuit of the underlying molecular events mediating NCAM's effects on synaptic transmission, Landmesser and colleagues confirmed in normal mice that synaptic vesicle cycling is regulated differently in immature and mature nerve terminals. Immature terminals use nifedipine-sensitive L-type Ca^{2+} channels to regulate release of neurotransmitter, and synaptic vesicle reformation is inhibited by brefeldin A. Transmitter release in mature terminals relies instead on P/Q-type Ca^{2+} channels, which are blocked by ω-conotoxin TK. Mature terminals have few L-type channels, and their clathrin-mediated vesicle recycling is relatively insensitive to brefeldin A treatment. The immature release mechanisms are fully replaced by the mature apparatus during postnatal development in normal mice.

However, in NCAM-deficient mice, nerve terminals retained the immature transmission components throughout the presynaptic terminal, as well as in nearby regions of the preterminal axon. Furthermore, although the mature components of vesicle cycle appeared in NCAM-deficient terminals, they were not organized around active zones, as occurs in normal terminals. NCAM therefore appears dispensable for proper initiation of synapse formation, but plays an important role in organizing nerve terminal maturation.

Postsynaptic Differentiation

The primary function of the muscle endplate is to translate neurotransmitter binding into a large postsynaptic depolarization. To this end, postsynaptic differentiation at the NMJ involves the creation of subcellular domains and morphological features that enhance the muscle's response to neurotransmitter.

The cardinal feature of postsynaptic differentiation at the NMJ is the clustering of AChRs into a high-density plaque, located in the sarcolemma opposite the nerve terminal. Ultimately, more than a dozen additional synaptic proteins become co-clustered with AChRs in the postsynaptic membrane, in the overlying extracellular matrix, and in the underlying cytoskeletal matrix. Nevertheless, AChR clustering is the earliest definitive postsynaptic specialization that can be identified, consistent with the central role of AChRs in mediating the postsynaptic response to neurotransmitter. AChR clustering is also one of the easiest synaptic features to detect experimentally, as AChRs are specifically and almost irreversibly labeled by α-bungarotoxin (see Box 2). Studies of postsynaptic differentiation have therefore focused on the mechanisms by which motor nerves and muscles control AChR clustering.

BOX 2. Taiwan Banded Krait—*Bungarus multicinctus*

C.C. Chang, C.Y. Lee, and their colleagues identified and characterized α-bungarotoxin as a major bioactive component in the venom of the many-banded krait (*Bungarus multicinctus*; above) indigenous to Taiwan (Chang, 1963, Lee, 1972). In retrospect, this discovery profoundly influenced progress in molecular, cellular, and developmental neurobiology. Due to the toxin's specificity and extremely high affinity for the nicotinic AChR expressed in vertebrate skeletal muscle, α-bungarotoxin became a key tool in its isolation and characterization. The nicotinic AChR was, therefore, the first neurotransmitter receptor and ion channel to be molecularly dissected. Fluorochrome-conjugated α-bungarotoxin has remained the primary means of identifying and monitoring the molecular and cel-lular differentiation of the neuromuscular synapse since its introduction in the mid-1970s (Anderson, 1974).

Postsynaptic differentiation is dependent on signals secreted by the nerve. Indeed, it has long been thought that sites of postsynaptic differentiation in muscle are determined by extrinsic signals secreted by axons where they contact developing muscle fibers. While this view continues to have great merit, recent studies indicate that muscle fibers possess an intrinsic program of postsynaptic differentiation, which is capable of forming rudimentary postsynaptic specializations without signals from motor neurons. To complicate matters further, innervation also provides a second, apposing signal that causes the disassembly of postsynaptic specializations. This inhibitory signal likely serves to eliminate secondary sites of postsynaptic differentiation along the muscle surface. We consider the central features of these mechanisms, below. One of the main tasks in the future will be to reconcile these separate programs of postsynaptic organization.

Extrinsic Control of Postsynaptic Differentiation

By monitoring the clustering of AChRs in cocultures of motor neurons and muscle fibers, Anderson and Cohen, and Frank and Fischbach, found that motor neurites promote postsynaptic differentiation at sites where they contact myotubes (Fig. 13) (Anderson and Cohen, 1977; Anderson *et al*., 1977; Frank and Fischbach, 1977, 1979). In contrast, sites of postsynaptic differentiation preestablished by the myotube are disassembled (Kuromi and Kidokoro, 1984). These observations provided strong evidence that neurons present signals that cue postsynaptic differentiation in the muscle, but did not provide an easy means of identifying them. In search of the source of the signals, Burden, Sargent, and McMahan studied the clustering of AChRs in regenerating muscle fibers, *in vivo* (Burden *et al*., 1979). They found that the synaptic basal lamina was capable of directing postsynaptic differentiation, even in the absence of a nerve terminal (Fig. 15). These experiments were similar to the empty basal lamina experiments used earlier to investigate presynaptic differentiation, but in this case, the muscle was allowed to regenerate within the original sheath of myofiber basal lamina, while the nerve was ligated to prevent reinnervation. Importantly, focus was newly directed at the composition of the synaptic basal lamina as a potential source of synaptogenic cues. McMahan and his colleagues identified a component that organized AChR clustering, which they called agrin. In a parallel series of studies, Fischbach and his colleagues identified a distinct basal lamina component that increased the levels of AChRs synthesized by the muscle fiber, which they named ARIA (for *a*cetylcholine *r*eceptor-*i*nducing *a*ctivity), and which was later identified as an isoform of neuregulin. Together, these studies demonstrated that nerve-derived signals can play dominant roles in the control of postsynaptic differentiation.

Lines of investigation leading from these discoveries ultimately identified three nerve-to-muscle signaling axes that control postsynaptic differentiation: Agrin-dependent activation of the muscle specific receptor tyrosine kinase, MuSK; neuregulin activation of ErbB receptors; and acetylcholine-mediated neurotransmission.

Agrin

McMahan and his colleagues hypothesized that axons induced local postsynaptic differentiation in the muscle by means of a secreted molecule, which was stably incorporated into the synaptic basal lamina at the mature NMJ. They identified this active component through biochemical purification, using Torpedo electric organ as a starting material rich in synaptic basal lamina, and the clustering of AChRs on cultured myotubes as a bioassay of postsynaptic differentiation. At each step of the purification, a sample of each fraction was added to the myotube culture medium; after a few hours, the cultures were stained with rhodamine-conjugated α-bungarotoxin. Active fractions caused a significant increase in the number of "hot spots" on the myotube surface, clusters of stained AChRs that appeared similar to the plaques of AChRs present at nascent postsynaptic sites *in vivo*. The name agrin given to the purified factor recalls the aggregation of surface AChRs that occurs with its addition to myotube culture medium.

Agrin is produced from a single gene as a large secreted heparan sulfate proteoglycan molecule (Rupp *et al*., 1991; Tsim *et al*., 1992). The polypeptide core contains nearly 2,000 amino acids arranged into distinct domains (Fig. 18). The N-terminal portion of the sequence contains conserved sites for attachment of the long glycosaminoglycan chains that make agrin a heparan sulfate proteoglycan, as well as several sites for asparagine-linked glycosylation. The N-terminus of agrin also contains follistatin repeats that are homologous to Kazal protease inhibitor domains. The C-terminal half of agrin contains three LG-domains, which are homologous to globular domains first recognized in the laminin α-subunit. Agrin's interaction with cells is likely dominated by its G-domains, which contain the AChR-inducing

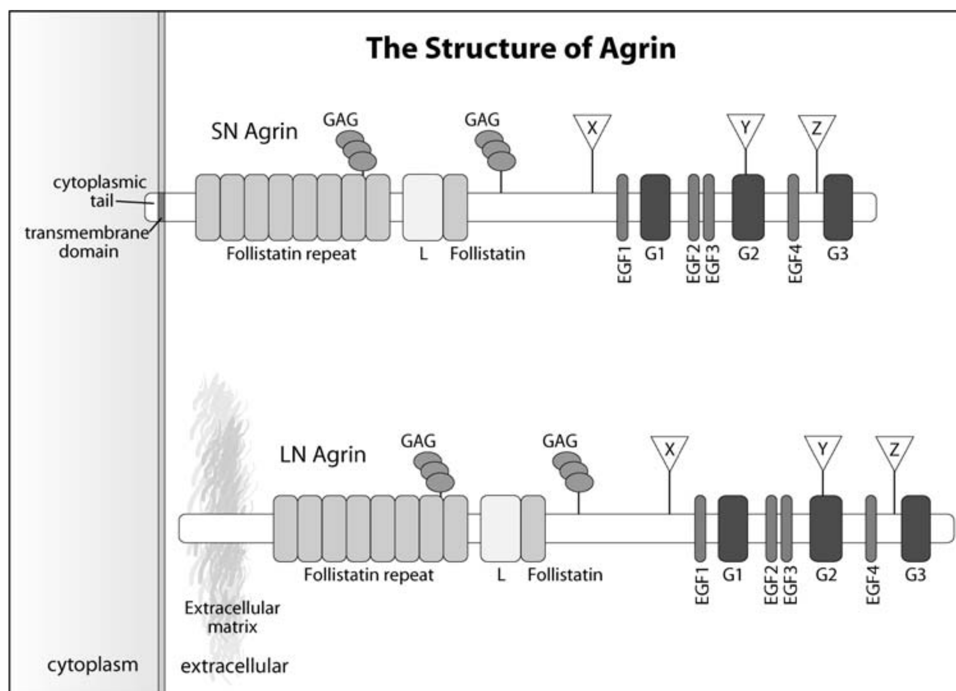


FIGURE 18. Agrin structure/function. Agrin is a large (*ca*. 400 kDal) heparan sulfate proteoglycan. The core polypeptide contains nearly 2,000 amino acids, most of which are present in recognizable domains. There are nine follistatin-like repeats in the N-terminal half of the protein; these bind growth factors and have protease inhibitor activity, but have uncertain biological roles at synapses. Heparan sulfate glycosaminoglycan chains are attached to serine or threonine residues near the middle of the protein. The C-terminal portion of agrin contains four epidermal growth factor (EGF)-like repeats, and three laminin-like G-domains that serve as ligands for the multifunctional matrix receptor dystroglycan. Among three conserved sites of alternative splicing (X, Y, Z) in the C-terminus, one dominates agrin's biological activity in postsynaptic differentiation. Variants with exon inserts at the Z site are up to 100-times more potent at causing ACh receptor aggregation on cultured muscle cells than variants lacking Z exons, and mouse embryos engineered to lack only the agrin Z-site exons fail to maintain postsynaptic sites during innervation. Agrin transcripts incorporating Z exons are found only in the nervous system; their absence in muscle cells explains why muscle-derived agrin is impotent at clustering ACh receptors. An additional transcriptional mechanism that may regulate agrin signaling is variation at the N-terminus. The SN form of the protein contains an N-terminal transmembrane domain, which is restricted to the nervous system and presumably tethers agrin to the neuronal surface (as a type II membrane protein). The LN form of agrin, which is produced from an alternative start site, contains an N-terminal laminin-binding domain in place of the transmembrane domain. The LN isoform of agrin is secreted by motor neurons and is concentrated in the synaptic basal lamina at the neuromuscular junction (NMJ), presumably through interactions with synaptic laminins.

activity, and which are ligands for the two major classes of matrix receptors in muscle cells, α-dystroglycan and β1-integrins. The incorporation of agrin into the synaptic basal lamina may rely on an identified interaction between N-terminal agrin domains and the laminin γ1-chain (Denzer *et al*., 1997, 1998).

McMahan's "agrin hypothesis" (McMahan, 1990) led to several predictions, including that motor nerves selectively synthesize agrin and secrete it during synapse formation, that agrin-induced signaling in the muscle fiber is sufficient to direct postsynaptic differentiation, that agrin and its intramuscular effectors are required for postsynaptic differentiation *in vivo*, and that agrin is concentrated in the synaptic basal lamina. These predictions have been tested and found accurate, although not without some interesting surprises.

Immunoreactivity for agrin is indeed concentrated in the synaptic cleft at the mature NMJ. However, an early quandary for the agrin hypothesis was that both neurons and muscle fibers synthesize and secrete agrin. In fact, agrin is abundant throughout the extrasynaptic basal lamina in developing muscle, a region that is not normally explored by the nerve. If muscles supply

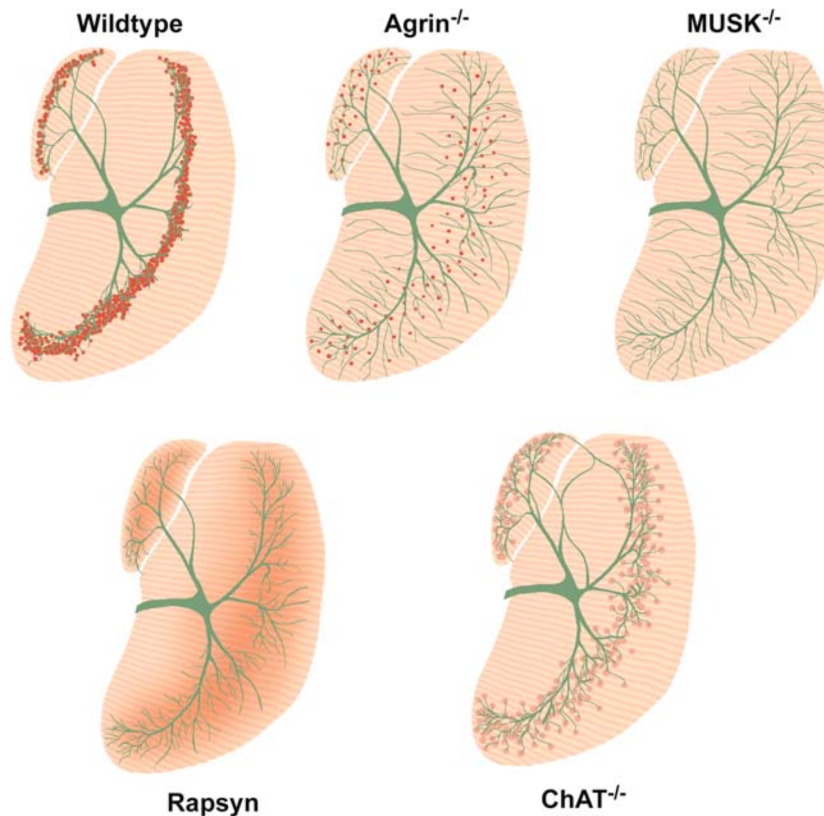


FIGURE 19. Comparision of synaptic defects in mutant mice lacking agrin, MuSK, rapsyn, and choline acetyltransferase (ChAT). Schematic diagrams show innervation of the left hemi-diaphragm, dorsal end up, in mutant mouse embryos genetically engineered to test the agrin pathway and the role of synaptic transmission in postsynaptic differentiation at the neuromuscular junction. In wild-type mice, the phrenic nerve contacts the developing diaphragm near its midpoint, and branches ventrally and dorsally; a medial branch extends to the crus, which attaches to the spine. Motor nerve terminals are located over AChR-rich postsynaptic sites, midway along each fiber, forming a so-called "endplate band." In agrin-deficient mice, the nerve initially enters and branches properly within the diaphragm, but ultimately grows far beyond the normal endplate. Few clusters of AChRs are present at perinatal ages, and these are broadly distributed and mostly unapposed by nerve terminals. The result is consistent with the hypothesis that agrin is required to establish stable postsynaptic sites. According to this view, axon overgrowth is secondary to the inability to establish synaptic contacts. No postsynaptic differentiation occurs in mice lacking the MuSK receptor tyrosine kinase. As in the absence of agrin, initial growth of the nerve is correct, but failure to terminate in the endplate region leads axons far into extrasynaptic muscle. The similarity of the MuSK and agrin phenotypes provides the strongest evidence that MuSK transduces the activity of nerve-derived agrin. Similarly in rapsyn-deficient mice, no clusters of AChRs form, and the nerve grows beyond the normal endplate region. However, AChRs are relatively enriched along the central portion of each fiber. The results support the idea that rapsyn mediates agrin- and MuSK-activated clustering of AChRs, but indicates that agrin/MuSK signaling (intact in these mice) promotes additional aspects of postsynaptic specialization that are rapsyn-independent, including elevated expression of AChRs. Additional studies support this notion, as there is a greater degree of postsynaptic transcriptional specialization in rapsyn mutants than in agrin- and MuSK-mutant mice. Correlation between the severity of nerve overgrowth and defects in postsynaptic differentiation supports the conclusion that axon outgrowth is normally inhibited by a retrograde signal associated with postsynaptic differentiation. Motor neurons in ChAT-deficient mice cannot synthesize ACh, preventing neurotransmission. AChR clusters on the muscle are individually larger, and collectively more broadly distributed. In part, this likely reflects an overabundance of motor axons, as there is less developmental apoptosis in the motor pools of ChAT mutant mice than in normal controls. The result suggests transmission, per se, is not required to establish neuromuscular junctions, but that nerve-evoked activity regulates the early pattern of synaptic connections in muscle.

agrin along their entire length, then how could axons employ agrin to specify the location of AChR clustering and postsynaptic differentiation? Would not muscle-derived agrin cause AChR clustering without contribution by the nerve?

Resolution came with the discovery that motor neurons produce an especially active isoform of agrin (Ruegg *et al*., 1992; Ferns *et al*., 1993; Hoch *et al*., 1993). The active isoform is encoded by specific mRNA splice variants, which are made by motor neurons and not muscle fibers. The locations of the alternative splice site sequences in the structure of the agrin polypeptide are shown in Fig. 18. The most important splice site has been named Z (or B, in avian transcripts). Splicing at the Z site involves two exons, encoding 0, 8, 11, or (the 8 + 11 combination) 19 amino acid residues in one of the G-domains. The Z+ isoforms (containing the 8, 11, or 19 residue inserts) are at least 100-fold more potent at clustering AChRs than the Z0 isoforms. Importantly, while neurons produce transcripts for active Z+ agrin, muscles produce only the inactive Z0 isoform of agrin. Thus, the paradox of agrin's distribution in developing muscle is resolved by tissue-specific expression and differential activity of alternative splice variants.

Targeted mutagenesis of the agrin gene in mice has established that agrin is essential for postsynaptic development *in vivo* (Gautam *et al*., 1996). Mutant mouse pups born without agrin are unable to move voluntarily, or even breath. Embryonic muscles and nerves develop normally without agrin, but fail to establish normal synaptic connections. At birth, muscle fibers in agrin-deficient mice have few AChR clusters, and little other molecular evidence of postsynaptic differentiation (Fig. 19). The motor axons also fail to form stable nerve terminals, and instead grow along the lengths of the muscle fibers, far beyond the normal region of axonal growth through the center of the muscle. Two observations suggest that defects in presynaptic differentiation in agrin-deficient mice are secondary to abnormal postsynaptic differentiation. First, nearly identical abnormalities are seen in mice lacking only the agrin gene's Z+ exons (Burgess *et al*., 1999). That is, despite an abundance of Z0 agrin produced by developing muscle fibers, muscle fibers fail to cluster AChRs, and motor axons fail to form stable nerve terminals, when Z+ agrin is specifically absent. Second, motor axons fail to form stable nerve terminals in mice lacking postsynaptic sites by other genetic mutations, despite completely normal levels of agrin. Thus, defects in presynaptic differentiation correlate strongly with absence of postsynaptic differentiation, but weakly or not at all with overall levels of agrin. While establishing a primary role for agrin in postsynaptic differentiation, these studies support the idea that postsynaptic sites are associated with a retrograde signal (not yet identified) that promotes presynaptic differentiation in the motor axon.

Agrin's activity in clustering AChRs is transduced by MuSK (Glass *et al*., 1996). MuSK is a prototypic member of the tyrosine kinase receptor family of transmembrane proteins. The single polypeptide contains an extracellular N-terminal domain with homology to the immunoglobulin superfamily, a single transmembrane segment, an intracellular protein tyrosine kinase domain, and several protein/protein interaction domains near the C-terminus. MuSK is concentrated at NMJs *in vivo* and co-clustered with AChRs on cultured myotubes. The application of agrin to myotubes in culture induces MuSK autophosphorylation. In other well-studied receptor tyrosine kinases, such as the EGF and PDGF receptors, autophosphorylation follows from ligand-induced dimerization and precedes binding and activation of downstream scaffolding and signaling components. These steps have not been well characterized in the case of MuSK. However, a number of experiments performed *in vivo* and *in vitro* have firmly established that MuSK is required for agrin-induced postsynaptic differentiation at the NMJ. First, MuSK and agrin are similarly required for postsynaptic development in embryonic mice (Fig. 19) (Dechiara *et al*., 1996). Second, myotubes cultured from embryonic MuSK-deficient mice are unable to cluster AChRs or other postsynaptic proteins in response to purified agrin. Third, activation of MuSK kinase activity by independent methods causes AChR clustering, and interference of MuSK catalytic activity blocks agrin-induced AChR clustering. These several criteria firmly establish that MuSK is an essential component of the agrin signal transduction pathway (Fig. 20).

One uncertainty in this model is how directly agrin interacts with MuSK. While full-length agrin binds and activates

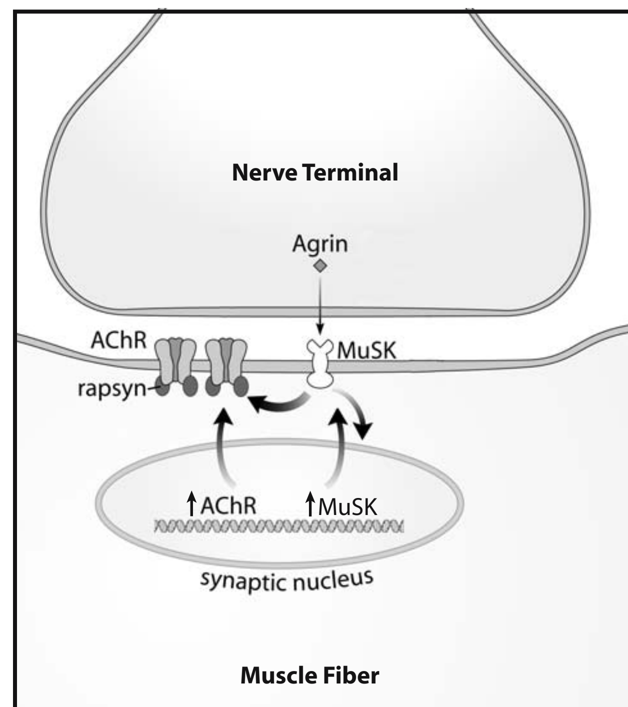


FIGURE 20. Agrin-induced signaling pathways. Motor terminals release Z+ splice variants of agrin (see Fig. 18). Z+ agrin activates MuSK concentrated in postsynaptic membranes. Autophosphorylation of MuSK is coincident with agrin-induced activation and may be prerequisite to the downstream activation of additional kinases leading to the clustering of acetylcholine receptors (AChRs). MuSK activation also increases expression of synapse-specific genes by subsynaptic myonuclei. Aggregation of AChRs on the cell surface occurs via the intracellular scaffolding protein rapsyn, likely through direct rapsyn–AChR binding.

MuSK (Parkhomovskiy *et al*., 2000), truncated versions of agrin activate MuSK and cause AChR clustering without detectably binding to MuSK. Thus, the binding and activation of MuSK by agrin have not been tightly linked, leading to speculation that an accessory factor may promote productive interactions between agrin and MuSK.

Signal transduction downstream of MuSK also remains unresolved. MuSK activation stimulates several intracellular signaling pathways, and a large number of known intracellular signal transduction kinases have been implicated, including Abl/Arg, Cdk5, FYN, GSK3, Src, and YES (Burden *et al*., 2002, 2003). Some are likely to activate small GTP-binding effector proteins, which promote actin remodeling and facilitate the structural changes that accompany postsynaptic differentiation. Others may mediate activation of the ras-mediated signaling pathway by which MuSK is known to regulate nuclear transcription. Several synapse-specific genes, including subunits of the acetylcholine receptor and MuSK itself, contain specific promoter sequences that mediate MuSK-induced upregulation. Auto-activation of MuSK expression provides a potential mechanism for positive feedback to concentrate MuSK signaling at developing synaptic sites. Finally, agrin/MuSK signaling also promotes the aggregation of ErbB receptors at the synapse, further influencing postsynaptic gene expression by mechanisms discussed below.

The best documented effector molecule downstream of MuSK activation is the 43 kDal AChR-associated protein known as rapsyn (Frail *et al*., 1988). Rapsyn is closely associated with the intracellular portion of AchR subunits and serves as a scaffold for receptor aggregation. Rapsyn itself has a complicated structure with several protein–protein interaction domains. Evidence in support of rapsyn's role include the observation that rapsyn and AChRs will spontaneously co-cluster when expressed together in fibroblasts (Phillips *et al*., 1991), and also the absence of AChR plaques or clusters in the muscles of rapsyn-deficient mutant mice (Gautam *et al*., 1995). Muscles in rapsyn-deficient mice also lack most other postsynaptic features, including synapse-specific gene expression.

The agrin/MuSK signaling axis, therefore, acts through rapsyn-mediated AChR clustering to scaffold the development of a postsynaptic apparatus (Fig. 20). Additional studies suggest that MuSK itself forms a primary scaffold on which AChRs and other postsynaptic components co-aggregate. While the discrete steps between the interactions of agrin, MuSK, and rapsyn remain unresolved, their definitive involvement in postsynaptic differentiation establishes a pathway by which motor axons play an essential role in regulating the time and place of synapse formation in the muscle.

Neuregulin

Postsynaptic differentiation includes transcriptional changes in gene expression. In muscle fibers, several nuclei are clustered immediately adjacent to the AChR-rich postsynaptic membrane. Genes for AChR subunits are transcribed at much higher rates by subsynaptic nuclei than by the nuclei that populate the rest of the muscle fiber. Fischbach and colleagues found that cultured muscle cells synthesized more AChRs when the culture media was supplemented with extracts of brain and spinal cord. They named the activity of the extracts ARIA, for *a*cetylcholine *r*eceptor *i*nducing *a*ctivity. In contrast to agrin, which aggregates AChRs already synthesized and present in the membrane, ARIA increased the levels of AChRs in the membrane but did not affect their distribution. The protein responsible for the ARIA activity was later purified and cloned, revealing an isoform of the intercellular signaling protein neuregulin. Purified and recombinant neuregulin has the same activity as ARIA, and antibodies to neuregulin and their erbB receptors selectively stain NMJs in skeletal muscles. Neuregulin upregulates the expression of AChR genes, one of the transcriptional hallmarks of postsynaptic differentiation. These results support the idea that neuregulin is a second postsynaptic differentiation signal provided by motor nerve terminals. Neuregulin's role in increasing AChR expression levels is complimentary to that established for agrin, which clusters AChRs already present in the membrane (Fig. 21).

ARIA activity is encoded by neuregulin-1 (NRG-1), one of four related neuregulin genes in mammals. NRG-1 is alternatively spliced to produce a set of related growth and differentiation factors, including glial growth factor, heregulin, and the neu differentiation factor, as well as ARIA (Lemke and Brockes, 1984; Holmes *et al*., 1992; Wen *et al*., 1992; Falls *et al*., 1993; Marchionni *et al*., 1993). NRG-1 signaling activity is associated with the epidermal growth factor (EGF) domain, which is present once in each isoform. This domain activates three members of the EGF family of membrane receptors, ErbB2, ErbB3, and ErbB4, which regulate cellular activities through intracellular protein tyrosine kinase domains. Receptor activation likely occurs through ligand-induced dimerization and autophosphorylation, as occurs with the closely related EGF receptor.

Motor neurons express multiple isoforms of neuregulin, which arise through transcriptional mechanisms. Muscle fibers express multiple erbB receptor subtypes, which are encoded by separate genes, but which form functional receptors as heterodimers. Confusingly, muscle fibers also express neuregulins, and Schwann cells express both neuregulins and ErbB receptors, offering a bewildering array of potential signaling interactions at the NMJ. The benefit underlying this high level of transcriptional complexity in NRG/ErbB signaling is not well understood.

One likely possibility is that the non-EGF polypeptide domains in neuregulin limit the range and specificity of neuregulin signaling. These domains vary considerably among NRG-1 isoforms. Some isoforms (types I and III) contain a transmembrane domain, which tethers the EGF-signaling domain to the cell surface. Neuregulin signaling in these cases may require direct cell–cell membrane interactions, as between the axon and Schwann cell. Alternatively, proteolytic cleavage at conserved sites proximal to the membrane can release the active EGF-domain into the extracellular space and across the synaptic cleft. Proteolytic release would provide a means of coupling neuregulin secretion to remodeling of the extracellular space. An additional complication is that alternative transcription start sites generate two N-terminal variants of NRG-1. One variant contains an extracellular Ig-superfamily homology domain; ARIA was

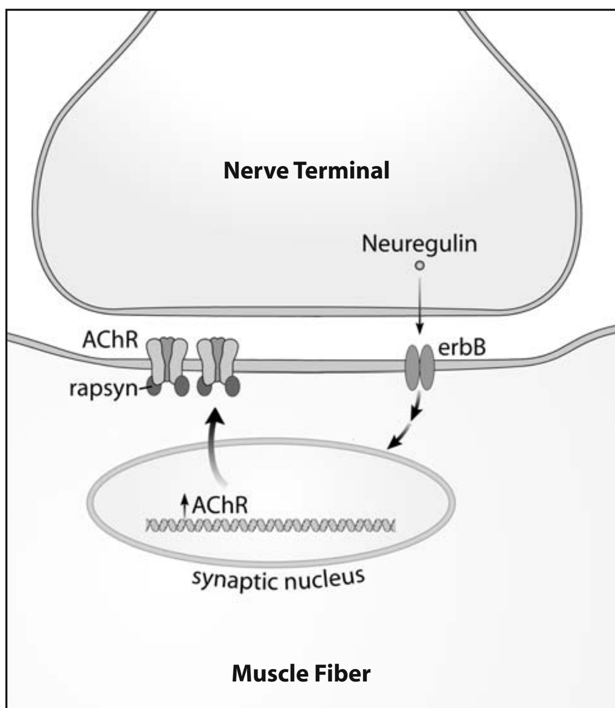


FIGURE 21. Neuregulin-induced postsynaptic signaling. Like agrin, neuregulin is concentrated in the synaptic basal lamina and is produced from alternatively spliced transcripts in neurons and muscles. In muscle fibers, neuregulin signaling activates ErbB receptors concentrated in the postsynaptic membrane. ErbB activation leads to an upregulation of AChR gene expression in subsynaptic myonuclei. Unlike agrin, neuregulin does not directly cause aggregation of AChRs.

originally identified in this form. A second N-terminal variant lacks the Ig-domain and instead encodes a cysteine-rich domain (CRD) coupled to a second likely transmembrane domain. Although the EGF domain remains extracellular in both N-terminal variants, two cleavage events are presumably required for the signaling domain to be released from the CRD-isoforms. The CRD-isoforms may therefore act primarily in cell-attached fashion. Consistent with this idea, studies in mutant mice indicate that the CRD-isoforms made by motor neurons act through ErbB2 receptors on Schwann cells to regulate Schwann cell survival (Wolpowitz *et al*., 2000). The requirement for neuron : Schwann cell contact in this signaling interaction may ensure that the number of Schwann cells required to myelinate the nerve matches the number and length of the developing axons; in this model, supernumerary Schwann cells lacking axonal contact fail to receive a neuregulin signal and die. Thus, through its varying domain structure, neuregulin may sometimes act as a paracrine factor signaling over short extracellular distances, or as a juxtacrine factor requiring cell–cell contact to transmit its signal.

Tests of neuregulin's role in synaptic development *in vivo* have produced mixed results. As mentioned, neuregulin produced by neurons is essential for the survival and proliferation of Schwann cells. In turn, developing motor neurons die in the absence of Schwann cells, hindering attempts to establish roles for NRG at synaptic sites. Nevertheless, four lines of evidence implicate NRG-1 in promoting the concentration of AChRs opposite the nerve terminal at the NMJ. First, treatment of cultured myotubes with purified and recombinant NRG selectively increases the levels of AChR subunit mRNAs, similar to the increased levels of subsynaptic receptor mRNAs observed *in vivo* (Harris *et al*., 1988; Martinou *et al*., 1991; Chu *et al*., 1995). Neuregulin also upregulates the expression of utrophin and voltage-gated sodium channels, which are concentrated at mature postsynaptic sites. Second, motor neurons synthesize, axonally transport, and secrete active NRG (Corfas *et al*., 1993). Third, NRG is present at developing NMJs, *in vivo*, and is concentrated in the synaptic cleft at mature NMJs (Goodearl *et al*., 1995). Neuregulin is stably associated with the synaptic basal lamina, possibly through interactions with heparan sulfate proteoglycans such as perlecan and agrin (Holmes *et al*., 1992; Loeb and Fischbach, 1995; Meier *et al*., 1998). This likely explains the observation that the synaptic basal lamina is able to direct synapse specific transcription in denervated muscles (Jo and Burden, 1992). Fourth, and most importantly, mice with reduced levels of one subset of NRG-1 isoform (those containing the Ig-domain) have strongly reduced levels of AChRs at the NMJ and are myasthenic (Sandrock *et al*., 1997).

Together, these studies suggest that neuregulin/ErbB signaling sustains the maturation of the postsynaptic apparatus by increasing the levels of synaptically abundant proteins as the size of the developing muscle fiber and the strength of the synaptic connection increase. An additional possibility is that neuregulin counteracts the effect of electrical activity in the muscle fiber; as we shall see in the next section, electrical activity reduces the synthesis of AChRs in extrasynaptic regions of the muscle.

Neuregulin signaling and agrin signaling neatly complement each other. Agrin appears to play a primary role in establishing the initial location and organization of postsynaptic differentiation. In contrast, neuregulin does not appear to be essential for establishing the site or pattern of synaptic gene expression, but rather supports the growth of the postsynaptic apparatus by amplifying the synthesis of postsynaptic components.

Acetylcholine

The third well-established signal at the NMJ is the neurotransmitter ACh. While the primary role of ACh is to open the cation-selective ion channel in the AChR and depolarize the muscle fiber membrane, ACh carries a second, longer lasting signal into the cytoplasm of the muscle fiber, in the form of calcium. The concentration of calcium inside the muscle fiber increases markedly during periods of electrical activity, as it does in most electrically excitable cells. In immature muscle fibers, this calcium comes principally from the extracellular medium through voltage-gated calcium channels in the sarcolemma. At synaptic sites, some calcium enters through AChR channels. In mature fibers, cytosolic calcium is rapidly infused through channels in the sarcoplasmic reticulum—an elaborate intracellular calcium storage/release system specific to muscle.

Calcium is well-established as a multifunctional second messenger. In muscle, of course, calcium triggers muscle fiber contraction by activating the myofibrillar actin–myosin complex. As in most cells, calcium regulates signal transduction pathways in muscle fibers, through the activation of protein kinases and phosphatases such as mitogen-activated protein kinase (MAPK), protein kinase C, calmodulin-dependent (CaM) kinases II and IV, and protein phosphatase 2B (calcineurin). These pathways, in turn, modulate the expression of specific genes, by regulating the function of transcriptional regulatory proteins, such as CREB and the basic helix–loop–helix (bHLH) factors. Synaptic transmission and muscle activity thereby have short-term and long-term effects on the development of the muscle fiber. Some of these effects contribute to the differentiation of postsynaptic and extrasynaptic regions of the muscle.

Muscle activity suppresses the expression of AChR subunits and other synaptic components in myonuclei throughout the extrasynaptic regions of the muscle (reviewed by Fromm and Burden, 1998). For example, cholinergic activity inactivates the gene for the AChR delta subunit through an E-box (CAnnTG) in the 5′-regulatory sequence. The E-box is a binding site for the muscle bHLH proteins MyoD, myogenin, MRF4, and myf5. This regulation is readily reversible, explaining why paralysis is accompanied by an upregulation of AChR levels in the extrasynaptic regions of muscle.

Since much of the electrical activity in muscles is driven by synaptic transmission, defects in synaptic function likely have secondary consequences for synaptic differentiation. One example where this seems very likely is in mutant mice lacking the gene for choline acetyltransferase (ChAT) (Misgeld *et al*., 2002; Brandon *et al*., 2003). Because ChAT is the sole enzyme responsible for the biosynthesis of acetylcholine from choline and acetyl-CoA, motor terminals in ChAT-deficient mice are unable to release ACh. Loss of synaptic transmission in ChAT-deficient mice does not prevent AChRs from clustering in the muscle membrane opposite the nerve terminal, which is consistent with the notion that agrin and neuregulin are secreted independent of ACh. However, loss of ChAT profoundly affects the distribution of synaptic sites in the muscle (Fig. 19). At birth, each muscle fiber normally contains one or a few centrally located synaptic sites. In ChAT-deficient mice, fibers appear to maintain as many as five or more AChR-rich postsynaptic sites, and these are spread along a much wider span of the muscle's length. The broader distribution of postsynaptic specializations is matched by a broader expression of synaptic transcripts in the muscle, and by increased formation of nerve terminals by the innervating axons. Qualitatively similar defects in the normal distribution of synapses occur in the muscles of chick embryos paralyzed by curare or bungarotoxin (Loeb *et al*., 2002). These observations appear to confirm the notion formulated during studies of nerve and muscle paralysis in polyinnervated adult muscle, that effective synaptic transmission suppresses the formation of secondary synaptic specializations and is required during development to restrict synapse formation to a single site in the muscle.

The primary conclusion to be drawn from these studies is that the muscle fiber responds to successful innervation in a way that makes it refractory to additional innervation. One attractive possibility is that secreted factors produced by uninnervated fibers to attract innervation by the nerve are downregulated upon initiation of successful cholinergic synaptic transmission. However, an equally plausible idea is that synaptic transmission increases the expression of retrograde inhibitory factors in extrasynaptic regions. While retrograde factors that promote or impede additional innervation have not been identified, it seems likely that their production is tightly coupled to cholinergic transmission. An additional surprise in ChAT-deficient mice is that muscles have significantly fewer muscle fibers, suggesting that ACh and/or muscle activity has an important role in myogenesis.

In summary, motor nerves secrete three factors that together serve to organize postsynaptic differentiation in skeletal muscle (Fig. 22). Agrin plays a primary role in establishing postsynaptic differentiation opposite the nerve terminal. Neuregulin sustains postsynaptic specializations as they mature. ACh, likely acting through changes in intracellular calcium in myofibers, suppresses ectopic innervation and thereby fosters the high degree of synaptic specificity present in mature muscle.

Intrinsic Control of Postsynaptic Differentiation

One prediction of the preceding model of neurally controlled postsynaptic differentiation is that uninnervated muscles should bear little evidence of postsynaptic organization. It was therefore surprising to find that robust postsynaptic differentiation occurs in muscles lacking nerve contact. Historically, this question has been addressed in mutant animals lacking proper innervation, such as the peroneal muscular atrophy mutation in mice (Ashby *et al*., 1993), in which a branch of the sciatic nerve fails to form and a muscle group develops without innervation. However, the patterns of innervation and synapse formation at embryonic ages are more readily addressed in the diaphragm, which develops earlier than many other muscles, and which is thinner and more readily stained as a wholemount preparation. Fortuitously, mutations in topoisomerase IIβ and the motor neuron transcription factor HB9 have been found to prevent the phrenic nerve from forming. The diaphragm in such mutants is never contacted by motor neurons (Yang *et al*., 2000, 2001; Lin *et al*., 2001).

The most intriguing observation in aneural embryonic muscles is that the AChRs aggregate in plaques, and the plaques are located in the central portion of the muscle (Fig. 23). Moreover, the uninnervated AChR-rich plaques contain many of the molecular specializations of normally innervated postsynaptic sites, including an AChE-rich synaptic basal lamina, and increased expression of AChR subunit genes by the underlying myonuclei. Clearly, muscle possesses an *intrinsic* program of postsynaptic differentiation.

At first glance, properly organized postsynaptic specialization in the absence of a nerve is contrary to the notion that nerve-derived agrin organizes postsynaptic differentiation. Indeed, the findings in aneural muscles are in striking contrast to the postsynaptic defects initially reported in mice missing either agrin or MuSK, where there is little or no postsynaptic

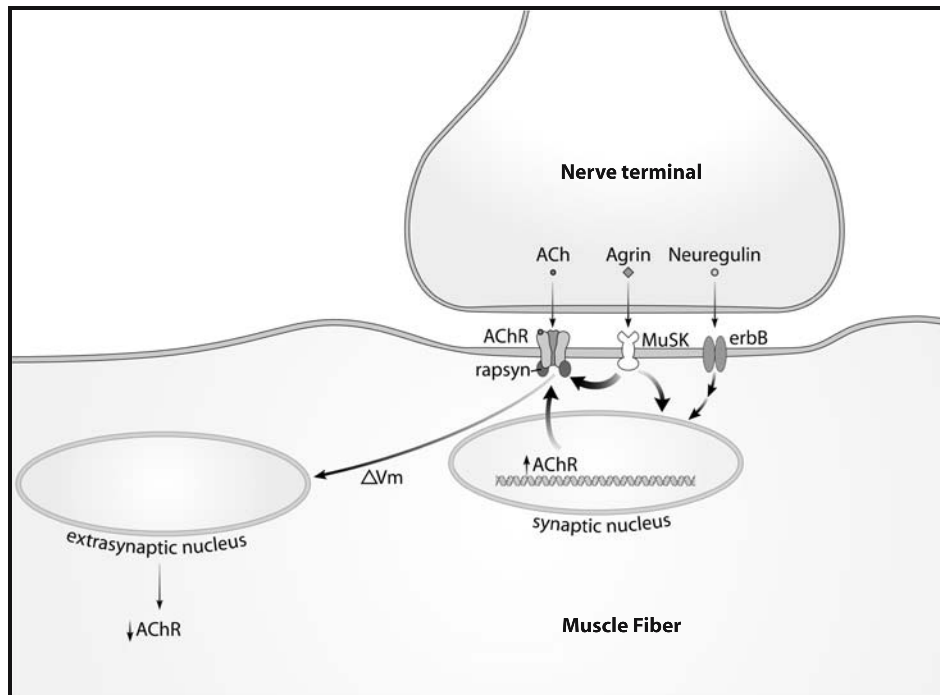


FIGURE 22. Agrin, neuregulin, and cholinergic transmission combine to control postsynaptic differentiation in muscle. Agrin promotes the concentration of AChRs and scaffolding proteins in the postsynaptic membrane. Neuregulin and agrin both amplify gene expression in postsynaptic nuclei, to enhance postsynaptic responses to ACh during growth and reorganization of the synapse. Nerve-evoked activity, mediated by the neurotransmitter ACh, suppresses the expression of synaptic genes by myonuclei in extrasynaptic regions of the muscle. Effects of muscle activity occur largely through increases in intracellular Ca^{2+}, which regulate a myriad of signaling pathways regulating gene expression and cellular activity.

differentiation. How are these observations to be reconciled? Close comparison of postsynaptic differentiation in normal, aneural, agrin-deficient, and MuSK-deficient animals yields several clear suggestions.

First, MuSK is required for all postsynaptic differentiation. No AChR plaques form in MuSK-deficient mice, with or without innervation. In fact, intrinsic postsynaptic differentiation is very sensitive to the levels of MuSK expression and is acutely affected by gene dosage. In HB9 mutant mice also heterozygous for a defective MuSK gene, the number of AChR clusters was reduced by 95% compared to HB9 mutants that carried two normal alleles of MuSK. It is not known whether this sensitivity represents cooperativity in MuSK activation, or a threshold in forming a MuSK-dependent scaffold for postsynaptic assembly. However, as developing muscle fibers grow preferentially at their ends, the central portion of the muscle is the oldest and has had the longest time to accumulate MuSK receptors. It seems likely that MuSK auto-activation, a phenomenon common among tyrosine kinase receptors, would be concentrated where its levels are highest, in the center of the muscle. Thus, MuSK autoactivation might initiate cell-autonomous postsynaptic differentiation in this part of the muscle. This explanation would account for the location of the endplate band in uninnervated muscles, as well as its dependence on and dosage sensitivity to MuSK.

Second, nerves provide two competing signals for postsynaptic differentiation: one (agrin) promotes postsynaptic specialization immediately adjacent to the nerve terminal; a second signal (most likely ACh) promotes muscle activity and causes degeneration of postsynaptic specializations located away from the nerve terminal. Reexamination of agrin-deficient mice at very early stages of innervation (at 13 days of embryogenesis, or about 6 days before birth) found that muscles initially contain a near-normal number and density of AChR-rich postsynaptic sites. These are presumably the muscle's intrinsic sites, now observed for the first time in an innervated (but agrin-less) muscle. The key observation is that these AChR-rich sites persist in muscles lacking a nerve (as e.g., in HB9-deficient mice) but disappear within a couple of days when the host muscle is innervated by agrin-deficient nerves. To be clear: In the absence of agrin, the nerve promotes *disassembly* of intrinsic postsynaptic sites.

Therefore, a more accurate assessment of neural-agrin's role in normal development may be that it acts to stabilize the intrinsic postsynaptic sites from activity-induced disassembly. Alternatively, neural-agrin could organize entirely new sites where it is secreted by the nerve, while the muscle's intrinsic sites are simultaneously disassembled. In fact, this replacement model is most consistent with original observations of nerve-induced postsynaptic differentiation, in nerve–muscle cocultures (Anderson and Cohen, 1977; Frank and Fischbach, 1979). As described earlier, cultured myotubes form spontaneous clusters of AChRs, which are eschewed by the neurites of cocultured motor neurons. Rather, motor axons organize new AChR clusters

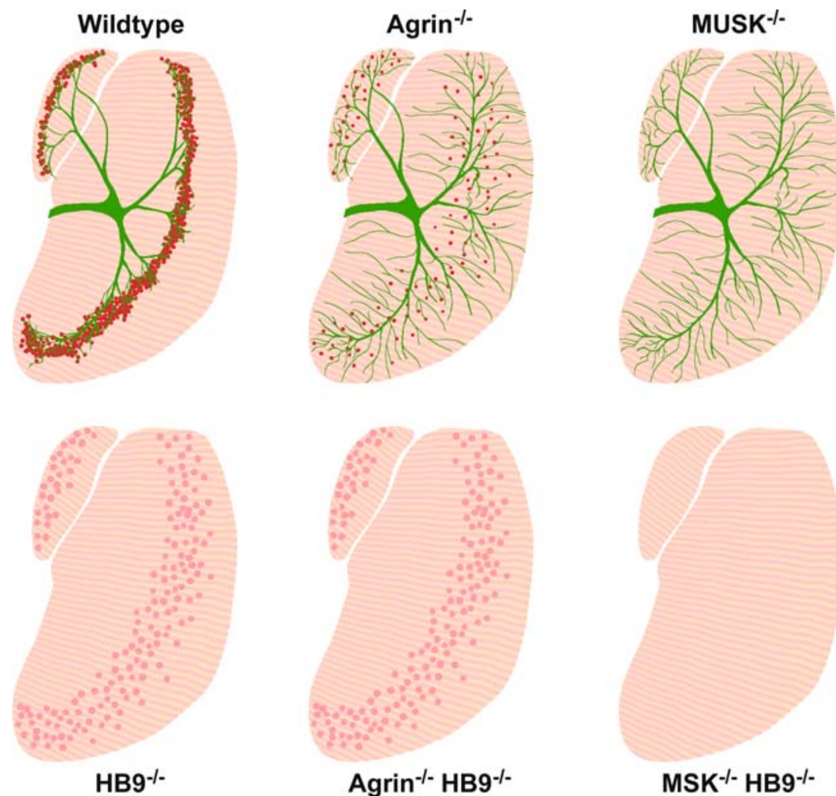


FIGURE 23. Antagonistic effects of agrin and innervation on postsynaptic differentiation in muscle. Schematic diagrams of innervation of the left hemidiaphragm in normal and mutant mice, as described in Fig. 19. Top panels reiterate dependence of postsynaptic specializations on agrin/MuSK signaling. Bottom panels illustrate postsynaptic differentiation occurring in the absence of innervation. In mice lacking the transcription factor HB9, cervical motor neurons that should innervate the diaphragm are absent. Interestingly, diaphragm myofibers in $HB9^{-/-}$ mice form plaques of AChRs near their midpoints, creating a rudimentary endplate band of postsynaptic sites in the absence of innervation. A similar distribution of postsynaptic sites is present in agrin-deficient mice, at very early stages of synaptic development. At late embryonic ages, postsynaptic differentiation in $HB9^{-/-}$ mice actually exceeds that in $agrin^{-/-}$ mice. The comparisons reveal that (1) muscle fibers have an agrin-independent, autonomous program of postsynaptic differentiation; (2) innervation provides a dispersal factor, which eliminates autonomous AChR clusters when agrin is absent; and (3) agrin/MuSK signaling is required to stabilize postsynaptic sites against nerve-induced dispersal. Muscle activity stimulated by release of ACh from motor axons may promote dispersal of any AChR clusters that are not stabilized by agrin. As autonomous AChR clustering is absent in the $MuSK^{-/-}$ background (i.e., in $HB9^{-/-}$; $MuSK^{-/-}$ double-mutant mice), it seems likely that autonomous clustering involves the activation of the MuSK signaling pathway at some point downstream of agrin. The results raise some uncertainty about the precise role of agrin, and whether motor axons or muscle fibers determine synaptic sites. If nerves innervate autonomous sites of AChR clusters provided by the muscle fibers, then agrin's role may be more of a maintenance factor. Alternatively, if nerves ignore autonomous clusters, then agrin both initiates and maintains permanent synaptic sites. This view is most consistent with observations in nerve–muscle cocultures, described in Fig. 13. Regardless, the complementary effects of agrin-induced clustering and activity-dependent dispersal of ACh receptors ensure that mature nerve terminals and endplates are aligned.

at sites of contact with the myotube, while the spontaneous AChR clusters are disassembled.

There remains, then, some uncertainty about the precise role played by the muscle's intrinsic program of postsynaptic differentiation. In principle, intrinsic postsynaptic sites may be selectively innervated *in vivo*, or they may be ignored and fully replaced. An intermediate possibility is that intrinsic sites are stabilized if the axon happens to arrive directly at their location, but that they are otherwise disassembled by activity-dependent mechanisms as new agrin-induced specializations are established at adjacent sites on the myofiber. A fourth possibility is that the initial set of postsynaptic specializations are not innervated immediately, but are associated with the production of retrograde signals that attract and induce the growth and presynaptic differentiation of the motor axon. Attempts to distinguish these possibilities by close observation in wild-type animals, during the very earliest stages of neuromuscular innervation (at about E13 in a mouse, or six days before birth), reveal that a large percentage of the initial complement of AChR clusters have in fact no direct nerve contact. Perhaps intrinsic postsynaptic sites are not directly targeted by motor axons. Nevertheless, because many nerve endings do co-localize with early AChR plaques in the very same muscles, these possibilities are not currently resolvable. In the absence of a dynamic view of initial innervation *in vivo*, the replacement model initially identified *in vitro* remains the most likely mechanism.

The vertebrate NMJ is the best studied synapse. We have focused here on a few well-established molecular events that direct

and define its formation, but myriad other signals undoubtedly contribute. Examples include growth factors and matrix proteases. Glial-derived neurotrophic factor (GDNF) perturbs NMJ formation when overexpressed in transgenic mice (Nguyen *et al*., 1998). Matrix metalloprotease 3 (MMP3) is concentrated at synaptic sites, where it is capable of releasing agrin from the synaptic basal lamina, and could play a role in synaptic remodeling or the dispersal of uninnervated postsynaptic sites (Vansaun and Werle, 2000). Despite years of study and real progress at the NMJ, a great deal remains to be learned about the complex interactions of axon, target, and glial cell at this best understood synapse.

CNS SYNAPSES

Compared to the NMJ, synapse formation in the CNS is poorly understood, for several reasons. CNS synapses vary considerably in function and specificity, but relatively little in size and structure. In addition, the complex anatomical architecture of the brain has hindered the ability to identify either a single axon's presynaptic terminals, or the postsynaptic specializations associated with a single dendrite. Even within topographically mapped populations there are numerous functional subtypes, such as the "On" and "Off" retinal ganglion cells in the eye, which so far lack molecular or anatomical features of distinction. Next, it is hard to observe one CNS synapse even twice, in search of changes that occur with development or use. Finally, there has been no CNS ortholog of the Torpedo electroplaques that would allow the unique molecular signature of a specific type of CNS synapse to be identified by biochemical means. Perhaps it should not be surprising that no clear kingpin of CNS synapse formation has been identified. Nevertheless, while the mechanisms of CNS synaptogenesis are relatively unknown, there are many functional analogies and some direct commonalities between neuromuscular and central synapses. One emerging theme is that synapse formation in the CNS includes a higher degree of functional redundancy and overlap than found at the NMJ, possibly reflecting the fact that any given neuron in the brain is a target for many hundreds of other neurons, often of several subtypes employing different transmitters.

To understand the requirements of synaptogenesis in the CNS, we first consider how synaptic transmission in the CNS resembles and differs from the NMJ. We then review mechanisms of synaptogenesis in the CNS, insofar as data support their role. Points of significant homology to or departure from well-understood events at the NMJ will be considered in course.

Structure and Function at Central Synapses

As at the NMJ, the control of neurotransmitter release at interneuronal synapses relies on presynaptic morphological and biochemical specializations in the axon, usually concentrated in small domains located at an axonal branch tip. Release of transmitter is commonly focused by active zone complexes, which are visible in electron micrographs as thickened (electron dense) segments of the presynaptic membrane that accumulate synaptic vesicles. SNARE complexes mediate docking and fusion of synaptic vesicles with the nerve terminal plasma membrane and trigger neurotransmitter release in response to elevated intracellular calcium. Fusion is followed by recovery and recycling of vesicle membrane components, enabling nerve terminals to function far from the cell nucleus. The molecular specializations supporting these functions (e.g., synaptotagmin, synaptobrevin, SNAP25, munc18, dynamin, rab5, voltage-gated calcium channels) are often identical or nearly identical to those at the NMJ. Thus, central and peripheral synapses rely on similar cellular and molecular presynaptic specializations.

The essential postsynaptic features of CNS synapses are also familiar. Neurotransmitter receptors are highly concentrated in the postsynaptic membrane directly opposite the presynaptic active zones. Additional voltage-gated ion channels are often concentrated in the membrane adjacent to the neurotransmitter receptor density, amplifying neurotransmitter-induced currents in the same way Na_V^+ channels concentrated in postsynaptic folds augment ACh-induced postsynaptic currents at the neuromuscular synapse. CNS transmitter receptors are co-concentrated with an array of primary scaffolding proteins and secondary signal transduction components that help co-concentrate the postsynaptic components and likely translate the recent history of synaptic activity into changes in synaptic strength and structure. A further parallel with the NMJ is that ribosomal complexes are found at postsynaptic sites in neurons. These may allow synaptic activity to regulate the synthesis of the postsynaptic components by translating synaptically localized mRNAs, analogous to the proposed role for transcriptional specialization of synaptic nuclei in skeletal muscle. CNS synapses also employ neurotransmitter clearance and re-uptake mechanisms to terminate synaptic signaling. Finally, the nerve terminal and postsynaptic specializations are maintained in precise register across a narrow synaptic cleft, through interactions between cell-surface adhesion receptors. As emphasized at the NMJ, proximity between sites of neurosecretion and reception is required for specific and effective neurotransmission. In many fundamental respects, therefore, interneuronal and neuromuscular synapses are alike.

One of the most notable features of synaptic transmission in the CNS, and one of the most obvious differences with skeletal NMJs, is the remarkable heterogeneity in inter-neuronal synaptic chemistry. The majority of inter-neuronal synapses use neurotransmitters other than acetylcholine, such as glutamate, GABA, or glycine. As there are few exceptions to Dale's hypothesis that each neuron employs a single primary neurotransmitter, each nerve terminal contains a restricted set of biosynthetic enzymes and transporters appropriate to the neurotransmitter. The variety of transmitters and neuromodulators used among interneuronal synapses is supported by an even greater variety of postsynaptic signal transduction mechanisms. These include ligand-gated ion channels, heterotrimeric G-protein coupled receptors, and peptidergic receptors.

A second, relatively obvious feature of most CNS synapses is their comparatively small size (Fig. 5). Most interneuronal synapses encompass a few square microns, rather than hundreds, and successful synaptic transmission in the CNS typically

involves the release of transmitter from one or a few synaptic vesicles, instead of hundreds, and detection by a few dozen postsynaptic receptors, instead of tens of thousands. At many inter-neuronal synapses, nerve terminal depolarization fails to release transmitter more often than it succeeds. Some of these synapses could represent the persistence of immature synapses in the adult CNS. Alternatively, the stochastic nature of transmission at such synapses may be their fully developed form. Indeed, just as the certainty of synaptic transmission at the NMJ relies on elaborate pre- and postsynaptic specializations, the tuning of central synapses to successfully transmit with a certain probability rather than with uniformity seems likely to depend on a high order of synaptic specialization.

To be sure, the weakness of individual synaptic connections in the CNS is typically counterbalanced by a high density of synaptic sites; the surfaces of neurons are often almost entirely covered by nerve terminals. The postsynaptic neuron thus integrates many synaptic inputs, each small, some excitatory, and others inhibitory. One consequence of this convergence is that the contribution of each synapse to postsynaptic activity is weighted by its proximity to the site of action potential generation, usually the target cell's axon hillock. Thus, excitatory glutamatergic transmission at a synapse on a distal dendritic spine will ordinarily have less of an effect on the membrane voltage at the axon hillock than a similar synapse located downstream on a dendritic shaft, whose activity in turn can be readily nullified by inhibitory synaptic input to the perikaryon. Therefore, the degree of neuronal arborization and the number and distribution of synaptic connections are especially critical aspects of synaptic development in the CNS.

A final CNS departure is the synaptic cleft, which contains a proteinaceous material but lacks the basal lamina present in the synaptic cleft at the NMJ. Typically 20 nm apart, the pre- and postsynaptic membranes at interneuronal synapses are close enough to involve direct interactions between adhesion molecules in the opposed membranes. Thus, signals that promote and/or maintain synaptic differentiation may be integral components of the synaptic membranes, rather than secreted extracellular matrix components. Interneuronal synapses also lack postsynaptic folds. If folds are neuromuscular specializations that allow the massive release of ACh to rapidly dissipate, then their absence at interneuronal synapses may reflect the relatively small synaptic area and low level of transmitter release.

Development of CNS Synapses

The lessons of synaptic organization at the NMJ suggest that synaptic differentiation between neurons is dependent on an exchange of molecular cues. However, as CNS synapses are sites of direct contact between the membranes of their pre- and postsynaptic cells and lack the basal lamina that stably incorporates agrin, neuregulin, and laminin at the NMJ, it has seemed more likely that homo- and heterophilic cell-adhesion molecules play roles in establishing, aligning, and/or maintaining synaptic specializations in the brain. Important roles have been proposed for cadherins and the neurexin:neuroligin complex. Certainly, soluble secreted factors may also play roles, and several have been suggested to play important roles in establishing or modulating synaptic connections. We consider each in turn.

ADHESION PROTEINS

Cadherins and Protocadherins

Cadherins are a large class of cell-surface membrane proteins, originally named for their dominant role in mediating calcium-dependent cell–cell adhesion. Four subgroups are identified: classical cadherins, protocadherins, desmosomal cadherins, and atypical cadherins. Each member contains at least one extracellular "cadherin" domain, and most are single-pass type I transmembrane proteins. Of these four types, we will discuss below the CNS roles of classical cadherins and protocadherins (Fig. 24), which are the best characterized.

Classical cadherins contain five extracellular "cadherin" repeats, and a relatively small intracellular domain. Classical cadherins mediate intercellular adhesion through homophilic interactions, such that among mixed populations of cells expressing different cadherins, cells expressing the same cadherin self-associate. The classical cadherin intracellular domain interacts with catenins, linking cadherin-rich membrane domains to actin cytoskeletal dynamics, and gene expression.

In the CNS, cadherins are concentrated at synapses. They have received special interest as mediators of synaptic connectivity, in part because homoselective binding offers a possible explanation for how axons select appropriate postsynaptic targets (Fannon and Colman, 1996; Uchida *et al*., 1996; Takeichi *et al*., 1997; Shapiro *et al*., 1999; Yagi *et al*., 2000). The "labeled line" model for synaptic connectivity in the CNS suggests that synapses preferentially form between pre- and postsynaptic cells that express complimentary adhesion molecules, as an electrician would splice a red wire to another red wire. In principle, homophilic cadherin interactions could serve as adhesive "labels" to instruct proper connectivity. However, while neurons in common circuits do express the same cadherins, they often express multiple cadherins, and synaptic connections do form between neurons that express different cadherins. This does not rule out an important role for cadherins in CNS circuitry, but suggests that whatever codes may exist are not simply reliant on cadherins.

Additional studies suggest that cadherins impart some of the specificity of synaptic connections in the CNS. One such example is in the avian optic tectum, a laminated region of the brain that receives multiple axonal projections from the eye and other brain regions. Retinal ganglion cell axons terminate in three of seven tectal cell layers. The laminar specificity of retinal innervation is directed by molecular cues that variously attract or repel the ingrowing retinal axons. Cadherins are among the cell-surface proteins differentially expressed between retino-recipient and non-recipient layers. Experiments designed to selectively perturb cadherin function altered the normal lamina-specific

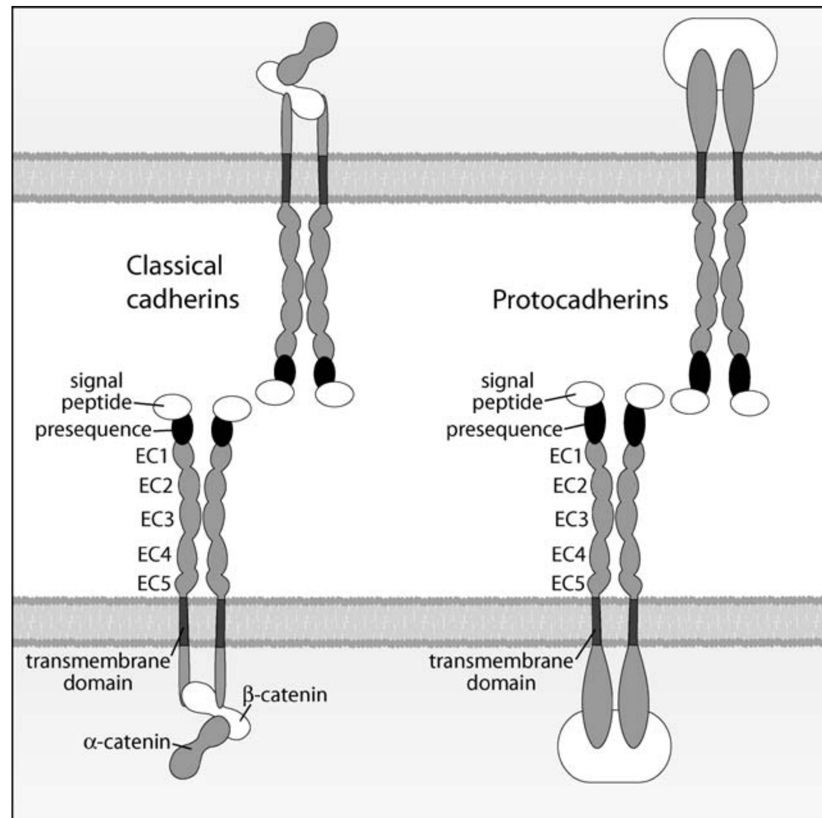


FIGURE 24. Cadherins and protocadherins. Classical cadherins are transmembrane proteins with modest intracellular domains and a series of five extracellular cadherin-specific domains. Cadherins play a significant role in promoting selective cell:cell interactions, through homophilic binding of specific cadherin isoforms. Intracellularly, classical cadherins bind to β-catenin, an important regulatory protein with links to both the actin cytoskeleton and to transcriptional regulation of gene expression. Protocadherins are similar to classical cadherins, but contain additional cadherin repeats. Intracellular interactions of protocadherins are less defined.

pattern of retinal innervation in the tectum (Inoue and Sanes, 1997). Other studies suggest that cadherins support cellular adhesion and molecular organization at synaptic sites. Detailed imaging found that cadherins are concentrated along the periphery of the synaptic densities, forming an adherens junction that surrounds the site of neurotransmission (Togashi *et al*., 2002) (Fig. 25). Thus, cadherins may act somewhat like a molecular zipper to bind initial pre- and postsynaptic specializations in precise registration.

Despite the attractiveness of these models for cadherin function in synaptogenesis, there is considerable uncertainty regarding the contributions of specific cadherin isoforms. Genetic perturbation studies in mice so far indicate that the formation of most synapses does not depend on an individual form of cadherin. For example, mice lacking cadherin-11 have mild abnormalities in CNS function, and no obvious morphological defects. In contrast, approaches that simultaneously inhibit multiple cadherins do alter synaptic structure. For example, dominant negative cadherin constructs that mimic the conserved intracellular domain of classical cadherins, and thereby compete for downstream intracellular cadherin-binding proteins, cause defects in the formation of dendritic spines (which are postsynaptic structures) in cultured hippocampal neurons (Togashi *et al*., 2002). These constructs presumably interfere with the downstream signaling from all of the classical cadherins expressed in these cells and thus have a broader effect than the inhibition of individual cadherins. One implication of the enhanced effect of interfering with multiple cadherins is that there is a significant degree of functional overlap between cadherins expressed in the CNS, or that specific not-yet-tested versions play dominant roles. It has not yet been possible to test some of the most obvious candidates for dominant roles, such as N-cadherin, which is expressed by many neurons. N-cadherin-deficient mice die from cardiac defects at mid-gestational ages, prior to the normal period of synaptogenesis. However, synaptic defects similar to those caused by dominant-negative cadherin expression result from loss of the adaptor protein αN-catenin, which mediates interactions with the intracellular domain of classical cadherins.

The protocadherins are a large family of cadherin-like cell-adhesion proteins, composed of dozens of related cell-adhesion proteins. Typical members possess six or more extracellular cadherin repeats, a single transmembrane domain, and an intracellular domain that is less well conserved than in classical cadherins (Fig. 24). The large number of protocadherin proteins is

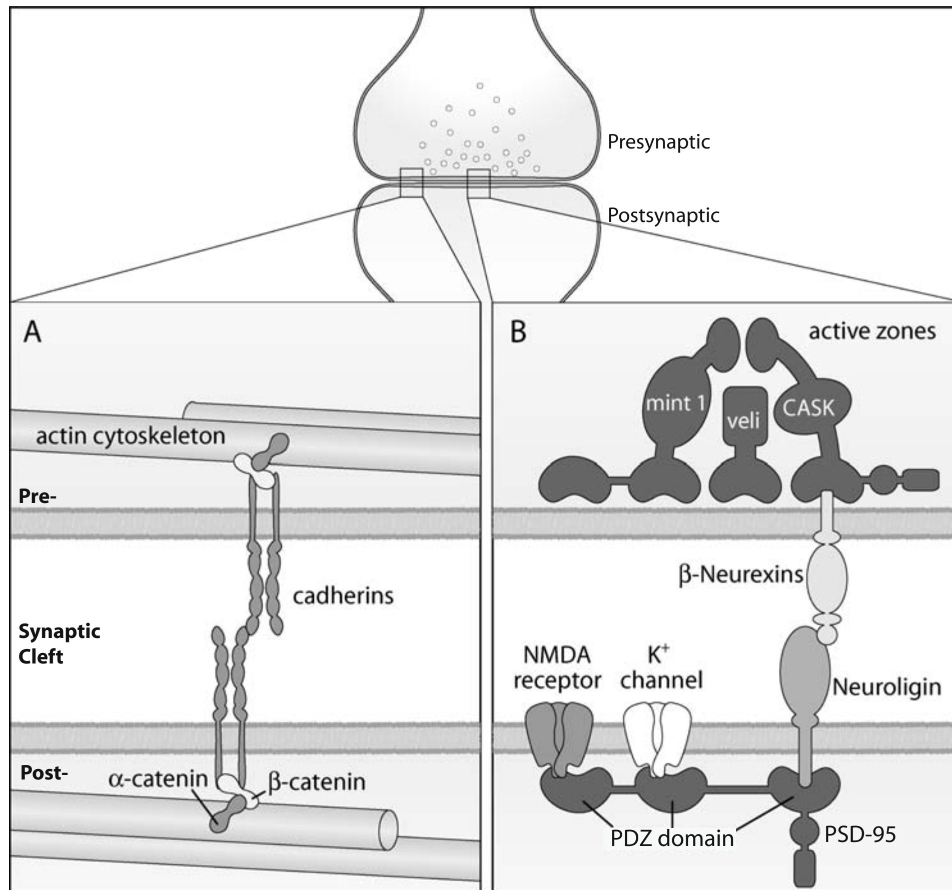


FIGURE 25. Synaptic adhesion complexes. (A) Cadherin complexes mediate homophilic adhesion. Cadherins are present at the borders of the presynaptic and postsynaptic densities, and interact with cytoskeletal elements within pre- and postsynaptic cells. (B) A second adhesion complex is formed by the interaction of β-neurexin with neuroligin, within the portions of the synapse involved in neurotransmission. Intracellular domains of both β-neurexin and neuroligin interact PDZ domains in synaptic scaffolding proteins. Presynaptically, β-neurexin interacts with the PDZ domain of CASK, which in turn interacts with veli and mint in the presynaptic density. Postsynaptically, neuroligin interacts with the PDZ domain of PSD-95, an integral component of the postsynaptic density. PSD-95 contains multiple PDZ domains, enabling it to link neuroligin to PDZ-binding neurotransmitter receptors and ion channels. Cadherins may serve to stabilize the adhesion of pre- and postsynaptic surfaces, and neuroligin/β-neurexin binding may serve to align the pre- and postsynaptic apparatus for neurotransmission.

partly a consequence of the genomic organization of their genes (Wu and Maniatis, 2000). Protocadherins are collected in three tandem gene clusters, termed α, β, and γ (Fig. 26). Within each cluster, the use of exons encoding the extracellular cadherin repeats, the transmembrane domain, and part of the cytoplasmic domain is highly variable; in contrast, exons encoding the remainder of the cytoplasmic domain are shared by all transcripts. This arrangement is generally similar to the arrangement of immunoglobulin genes and allows for a tremendous degree of diversity in the protein products. Such diversity would presumably be of tremendous value as a molecular array regulating synaptic specificity in the brain. However, the variable exon usage that produces individual protocadherins also hinders the study of individual variants. Moreover, deletion of the entire γ-protocadherin complex in mice results in neonatal lethality, and a great deal of apoptotic cell death in the nervous system (Wang *et al*., 2002). While neurons cultured from these animals form an initial set of synapses before rapidly dying, more refined perturbations will be required to understand whether synaptic abnormalities contribute to the excessive neuronal cell death.

Neurexin and Neuroligin

Neurexin and neuroligin are neuronal cell-surface proteins present at central synapses (Figs. 27 and 28). Unlike the cadherins, their interactions are heterophilic. Neurexins on the presynaptic cell bind to neuroligins and dystroglycan on the postsynaptic cell. Neuroligins preferentially bind β-neurexins, forming an especially tight complex. Much like cadherins, however, these interactions likely serve multiple roles in the CNS, quite possibly including the organization of new synapses and the stabilization of mature synapses.

Neurexins were identified in a search for the neuronal receptor for α-latrotoxin, a component of black widow spider venom (Ushkaryov *et al*., 1992). The α-Latrotoxin causes massive exocytosis of neurotransmitter by stimulating the unregulated

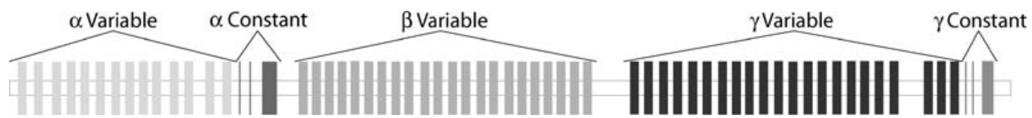


FIGURE 26. Genetic organization of protocadherin diversity. Synaptic membrane proteins with hypervariable domains are attractive candidates to mediate the specificity of synaptic connections. Variability among protocadherins depends primarily on alternative splicing. The α-protocadherins are produced from a single gene containing fourteen "variable" exons, which are spliced to form the five or six extracellular cadherin repeats found in these isoforms, and three "constant" exons, which encode the transmembrane and intracellular domains present in all α-protocadherins. The β-protocadherins are produced from twenty-two variable exons. The γ-protocadherins are produced from 3 constant exons, and 22 variable exons. Given the possible number of exon combinations, these genes are capable of generating an astounding array of protein isoforms. The arrangement of protocadherin genes in clusters is similar to immunoglobulins.

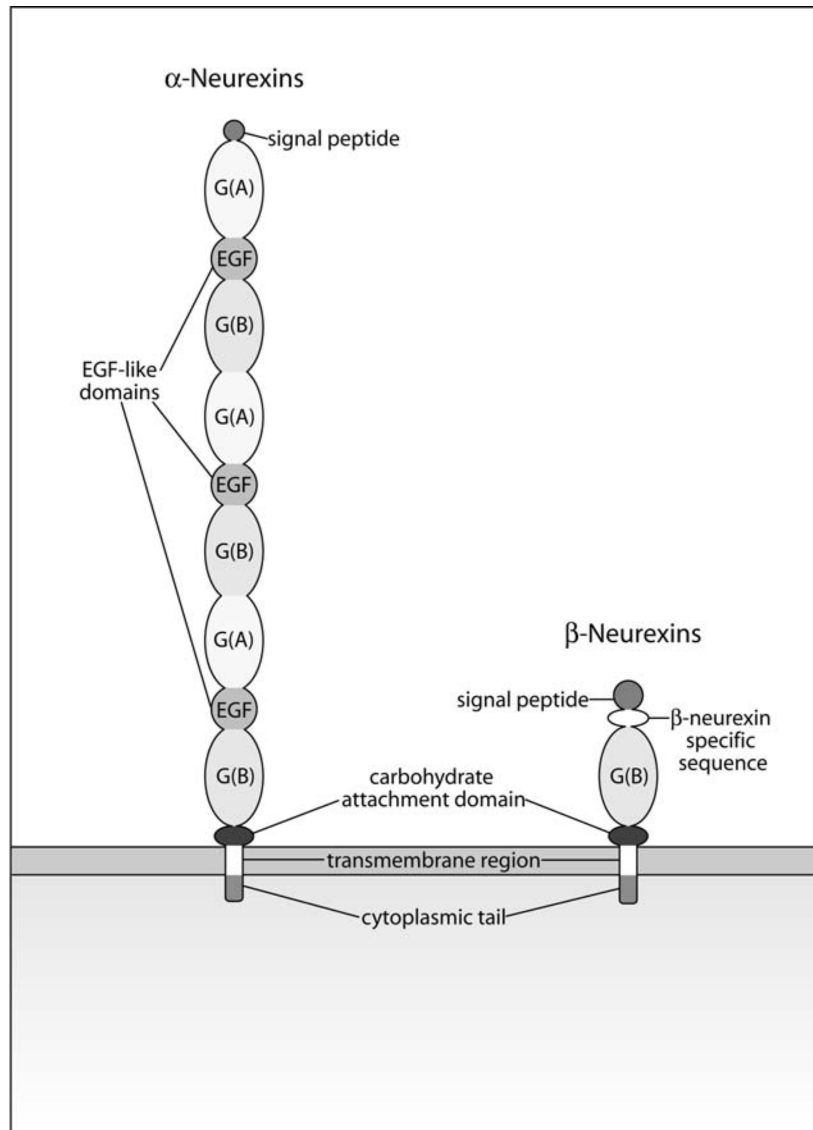


FIGURE 27. Neurexin structure. Neurexins are type I membrane proteins. Each contains a short cytoplasmic domain and a single transmembrane domain. The majority of neurexin mass is extracellular. The α-Neurexins contain 6 laminin-G domains and 3 EGF domains. Sequence similarities between the G-domains in α-neurexins suggest evolutionary triplication of an ancestral pair of G-domains across an EGF-like domain [i.e., G(A)-EGF-G(B)]. The β-neurexins contain a single G-domain and may represent a beneficial truncation of the ancestral α-neurexin G-domain pair. Considerable diversity in neurexin isoforms arises through a conserved splice site present in each G(B) domain. G-domains were originally named on their discovery in the α1-chain of laminin and have also been called LNS domains for their common appearance in laminins, neurexins, and the soluble hormone-binding S-protein. G-domains in agrin, perlecan, and laminin α-chains are ligands for receptors at the neuromuscular junction. The Z-splice site in agrin that regulates ACh receptor clustering is located within an agrin G-domain. Thus, through genetic duplication and alternative splicing, G-domains may have provided a common protein platform for organizing multiple aspects of pre- and postsynaptic differentiation across the synaptic cleft.

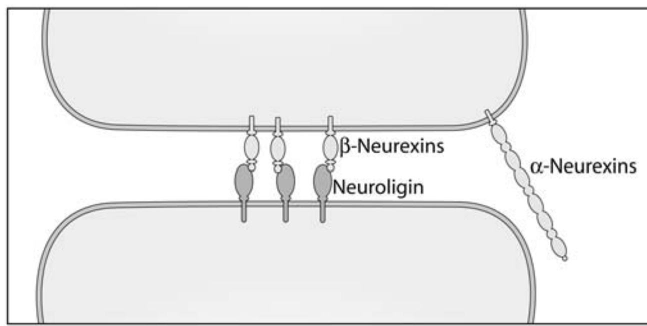


FIGURE 28. β-Neurexins, but not α-neurexins, interact with neuroligin across the synaptic cleft.

fusion of synaptic vesicles with the nerve terminal surface. The neurexin interaction with α-latrotoxin initially indicated that neurexin was not only present on presynaptic terminals, but in intimate association with the vesicle fusion machinery. This distribution has been difficult to confirm by conventional immunological methods, as antibodies to neurexins are poor. Nevertheless, transgenic mice concentrate neurexin-fusion protein epitopes at nerve terminals.

Neuroligins were identified by biochemical methods, as they bind directly and specifically to the β-neurexins. Antibodies specific for neuroligins readily label synaptic sites in brain, and staining with immunogold-labeled antibodies shows neuroligins specifically localize to the postsynaptic surface of the synaptic cleft.

The neurexin family is highly polymorphic. Gene duplication, multiple promoter elements, and alternative splicing produce a large number of potential neurexin isoforms. Neurons express neurexins from at least three genes (*Nrxn1*, *Nrxn2*, *Nrxn3*) (Missler *et al*., 1998). A fourth neurexin gene encodes a more distantly related protein, which is selectively expressed by glia. The *Nrxn1–3* genes each contain two independent promoters, which generate longer α-neurexins and shorter β-neurexins (Fig. 27). Five conserved splice sites decorate the α-neurexins; two of these sites are included in β-neurexins. As a result, there are nearly 3,000 potential neurexin isoforms. Like the cadherins and protocadherins, neurexin diversity is a tantalizingly diverse molecular resource and has been proposed to contribute to the molecular basis of synaptic specificity in the brain. Analyses of neuronal transcripts indicate that a considerable number of the possible neurexin variants are actually expressed in the mature nervous system.

Variability in the neurexin gene transcription is targeted to the extracellular polypeptide domains. Each *Nrxn* gene encodes a major extracellular domain, a single transmembrane domain, and a modest intracellular domain. The extracellular domain is dominated by regions of homology to the LG-domain. The α-Neurexins contain six LG-domains. The β-Neurexins are initiated from a second, downstream promoter, and include only the final LG-domain, nearest the transmembrane domain. The tertiary structure of the LG-domain has been determined (Hohenester *et al*., 1999; Rudenko *et al*., 1999; Timpl *et al*., 2000). Of the five conserved alternative splice sites, three are specifically targeted to exposed loops of the LG-domain.

Interestingly, there is a notable precedent where alternative splicing in the LG-domain is critically important to synapse formation. Laminin G-domains are relatively common structural elements in extracellular matrix proteins and are concentrated in the synaptic basal lamina of the NMJ. Five LG-domains are present in tandem at the C-terminus of the laminin α2-, α4-, and α5-chains, and three G-domains are present in agrin (Figs. 16 and 18). They often (but not always) serve as binding sites for dystroglycan (Fig. 11), a matrix receptor concentrated at synaptic sites in both the PNS and CNS. However, LG-domains are also associated with neuronal signaling properties. The G-domains in the eponymous laminin-1 heterotrimer contribute to neurite adhesion and growth cone motility. Moreover, the AChR clustering activity of agrin is due to an alternative splice variation in a loop of the third LG-domain in agrin. LG-domains have a 14 β-strand structure, in which two antiparallel β-sheets are layered against each other, like an empty sandwich. Loops connecting the β-strands rim the margins (like a sandwich's crusts). The loops are relatively unconstrained and readily accommodate sequence variations. Accordingly, the Y- and Z-splice sites in agrin alter small peptide elements in adjacent LG-domain loops; both variations control agrin's ability to activate the MuSK receptor kinase. Possibly, splicing in neurexin's LG-domains mimics that in agrin. Moreover, it varies among brain regions, raising the possibility that neurexin LG-domain splicing has functional relevance to the organization of synaptic circuits. It remains uncertain whether documented differences represent cell-specific splice variation, or how many isoforms may be expressed at synaptic sites. There is also little notion of how variation in neurexin splice isoforms is recognized by postsynaptic receptors, as neuroligins do not appear to present a similar diversity. Nevertheless, functional studies suggest neurexins are important elements of nerve terminal differentiation.

Brain function in mice lacking individual neurexin genes is mildly or little affected. In contrast, mice lacking two or three of the α-neurexin genes are strongly affected and most die within one week, with disruptions to the rhythms of breathing (REF). Loss of α-neurexins causes a marked decrease in calcium-dependent synaptic vesicle fusion and evokes neurotransmission at both inhibitory (GABA-releasing) and excitatory (AMPA-sensitive glutamatergic) synapses. Importantly, while calcium channels are expressed at normal levels and have normal intrinsic conductances in the absence of α-neurexins, the calcium channel current density decreases precipitously during the period of synapse formation, compared to normal controls. There is no detectable defect in synaptic structure in the absence of α-neurexins, although there is a selective loss of brainstem GABA-releasing nerve terminals, which could account for the defects in breathing. Together, the results demonstrate an important functional role for the α-neurexins and indicate that α-neurexins are target-derived signals that regulate the location and/or activity of presynaptic calcium channels at sites of neurotransmitter release. They do not, however, discriminate functional

differences between potential neurexin splice variants. These results also recall the previously described role of laminin-9 at the NMJ, which interacts specifically with presynaptic calcium channels and organizes the position of active zones in the nerve terminal membrane.

Mice lacking α-neurexins appear to express β-neurexins at normal levels. Additional studies suggest β-neurexins have important, but distinct functions at central synapses. First, the β-neurexins (one from each *Nrxn* gene) are specific trans-synaptic binding partners for neuroligins. Neuroligins are members of a gene family with at least three members in mammals. They are type I single-pass transmembrane proteins, with a single large extracellular domain that selectively binds β-neurexins. Alternative splicing of neurexin may alter this interaction, as incorporation of additional amino acid residues into the β-neurexin extracellular domain abolishes neuroligin binding. There also appears to be specificity through neuroligin expression; for example, neuroligin1 is excluded from GABAergic synapses. The extracellular domain bears strong sequence homologies to cholinesterases, but is catalytically inactive.

Second, *in vitro* studies have found that cultured neurons form presynaptic structures on non-neuronal cells that are transfected with constructs for recombinant neuroligins (Scheiffele *et al*., 2000). Little or no nerve terminal formation occurred on neuroligin-expressing cells when soluble β-neurexin fusion proteins were added to the culture medium. The results suggest that neuroligin interactions with axon-associated β-neurexins promote the formation of presynaptic specializations, including terminal varicosities, synaptic vesicle accumulations, biochemical differentiation, and active zone localization.

The mechanisms by which neurexin/neuroligin bindings are transduced into synaptic organization are not yet known. One possibility is that they serve primarily as synaptic adhesives, tying pre- and postsynaptic membranes together, with additional membrane protein interactions driving synapse assembly. Alternatively, the neurexins and neuroligins could serve as platforms for signaling or scaffolding proteins and thus play more active roles in directing or stabilizing synapse formation. In support of this latter idea, the cytoplasmic domains of neurexins interact with the PDZ domain protein CASK (PDZ domains are described in detail later), which ultimately links to the presynaptic release apparatus (Fig. 25). In a blessed fact of simplicity, each α- and β-neurexin isoform encoded by a given gene (*Nrxn1*, *2*, or *3*) has a common, invariant cytoplasmic domain. This could provides a mechanism to allow neurexins to directly connect diverse extracellular ligands (binding to the hypervariable neurexin LG-domains) to machinery of neurotransmitter release, which is shared at synapses throughout the nervous system. Similarly, neuroligins interact with the PDZ domain protein PSD95, which provide a direct link to the glutamate receptors and potassium channels concentrated at postsynaptic sites. Thus, by virtue of their localization, diversity, and extracellular adhesive properties, neurexins and neuroligins are attractive synaptogenic candidates at central synapses. Is summary, by simultaneously anchoring the anterograde and retrograde organization of synaptic protein complexes, neurexin/neuroligin interactions may promote the coincident formation of pre- and postsynaptic specialization.

Cadherin homophilic interactions and neurexin/neuroligin heterophilic interactions represent the best current view of CNS synapse formation. First, both are adhesion-based mechanisms that link extracellular interactions to intracellular signaling and protein localization. Second, each includes the potential for considerable molecular diversity, and they are therefore plausible candidate substrates underlying specificity in synaptic connections. Each may also play important roles in the nervous system beyond synaptogenesis. Cadherins are certainly involved in cell migration and the growth of axons and may be involved in neuronal survival as well. Neurexins and neuroligins seem well suited to regulate similar events before and after synaptogenesis. It is worth noting, however, that both sets of interactions are calcium dependent, while synaptic adhesion is not. Additional calcium-independent mechanisms of adhesion, such as immunoglobulin superfamily adhesion molecules, may therefore be essential components of synaptic interactions in the CNS.

SIGNALING FACTORS

Agrin and Neuregulin Play Uncertain Roles

Synaptogenesis at the NMJ relies on locally secreted cues passed between nerve and muscle. While agrin and neuregulins are obvious starting points in the search for similar controlling factors in the CNS, their roles there remain unclear. Several observations suggest agrin may promote the organization of synaptic specializations in the brain. Agrin is broadly expressed in the CNS, by many neuronal cell types in addition to cholinergic neurons. Much of the agrin expressed in the CNS is the Z+ isoform, which is "active" in clustering AChRs at the NMJ. Interestingly, unlike the NMJ, much of the agrin in the CNS is the product of an alternative transcriptional start site that creates an N-terminal transmembrane domain. This produces agrin as a type II transmembrane protein, in which the AChR-clustering signaling domain remains extracellular. Presumably, tethering agrin to the neuronal membrane represents a mechanism to anchor agrin to specific extracellular sites in the CNS, which lacks the semiautonomous form of extracellular matrix (the basal lamina) that pervades the PNS (Neumann *et al*., 2001; Burgess *et al*., 2002). Neurons are also capable of responding to agrin. In neuronal cultures, the addition of soluble agrin causes an increase in CREB phosphorylation and cFOS expression and alters neuronal morphology (Ji *et al*., 1998; Hilgenberg *et al*., 1999; Smith *et al*., 2002). More provocatively, antiagrin antibodies and transfection with agrin-specific antisense oligonucleotides perturb synapse formation between neurons in culture; synapse formation is restored by application of exogenous agrin to the culture medium (Ferreira, 1999; Bose *et al*., 2000; Mantych and Ferreira, 2001). Despite these supportive results, CNS development in agrin mutant mice appears relatively normal, and primary neurons cultured from these mice display few or no detectable defects in synaptogenesis (Li *et al*., 1999; Serpinskaya *et al*.,

1999). How can these disparate *in vivo* and *in vitro* results be reconciled? One possibility is that the *in vitro* environment for synapse formation is artificially simple, allowing a minor, modulatory role for CNS agrin to be magnified. A second, common explanation for the lack of a "knockout" phenotype is redundancy among related factors. While no other agrin-like genes have been identified, it could be that the relevant signaling domain in agrin is reduplicated in other gene products. Indeed, the LG-domains which incorporate agrin's synaptogenic activity at the NMJ are present (as inactive isoforms) in a broad array of extracellular proteins in the CNS as well as the PNS. One of these, of course, is neurexin, described in the previous section.

A specific role for neuregulins in synapse formation in the CNS is even more obscure than that for agrin. Neuregulin is a multifunctional signaling factor in the nervous system, with significant roles in the fate and migration of neural crest derivatives. These events are especially crucial to the development of the brain's cellular architecture. Thus, defects in other neuronal behaviors may obscure specific roles for neuregulins in synapse formation. While agrin and neuregulin have uncertain roles in synapse formation in the CNS, other secreted signaling molecules have received more direct experimental support. These include the WNT/wingless signaling pathway, and NARP.

WNT Signaling

WNTs are a family of vertebrate proteins with homology to wingless (Wg), a secreted cell signaling glycoprotein in *Drosophila*. As the *Drosophila* name implies, wingless was identified through mutations that disrupt wing development. In the best characterized function of WNTs, Wg is a *Drosophila* morphogenetic factor that establishes polarity in developing anatomical elements, such as the segments of the embryonic body and the imaginal discs that produce the adult body structures. Vertebrate WNT proteins act in similar fashion, as short range signaling factors. They play critical roles in neural and axonal development (Burden, 2000; Patapoutian and Reichardt, 2000).

WNT signaling activities are mediated by Frizzled (Fz) receptors, a family of membrane proteins also first identified in *Drosophila* (Fig. 29). Fz receptors have a domain structure related to the seven-transmembrane domain, G-protein coupled receptors. Low-density lipoprotein receptor-related proteins (LRPs), a family of single-pass membrane proteins, serve as essential co-receptors for WNTs. WNTs also bind to heparan sulfate proteoglycans, which may be important for establishing gradients of WNT in the extracellular space. The WNT downstream signal pathway is best studied in non-neuronal cells. Activation of Fz receptors leads to the phosphorylation of Disheveled (Dsh). Phosphorylated Dsh prevents ubiquitin-dependent degradation of β-catenin, a protein that promotes the expression of WNT-responsive genes. Phosphorylated Dsh stabilizes β-catenin indirectly, by disrupting the formation of a complex between glycogen synthase kinase 3β (GSK3β), the adenomatous polyposis coli protein (APC), and the scaffolding protein Axin. The assembled complex phosphorylates β-catenin, promoting its ubiquitination and degradation. Stabilized β-catenin is required for specific transcription factors (Lef/Tcf) to activate gene expression. In addition to affecting β-catenin, WNTs inhibit GSK3β-catalyzed phosphorylation of microtubules, thereby influencing cytoskeletal dynamics by increasing the stability of microtubule bundles.

Several studies indicate that WNT/Fz signaling is important during synaptogenesis. First, Wg/Fz signaling occurs at the *Drosophila* NMJ, and mutations in Wg cause defects in synaptic structure and function in *Drosophila* muscles (Packard *et al.*, 2002, 2003). The *Drosophila* NMJ is branched and varicose, like the vertebrate NMJ, but uses glutamate as neurotransmitter, like most excitatory synapses in the vertebrate CNS. Wg is secreted from motor neurons during synapse formation at *Drosophila* NMJs, where it activates myofiber Fz2 receptors. Mutations in Wg disrupt the normal postsynaptic aggregation of glutamate receptors and scaffolding proteins, as well as the elaborate structure of the postsynaptic membrane. Retrograde defects are also seen in Wg-deficient presynaptic boutons, which concentrate vesicles but lack their normal complement of mitochondria and presynaptic densities. It is attractive to consider that the presynaptic defects are a direct result of impaired microtubule-based trafficking in the absence of Wg. However, presynaptic defects could be secondary to impaired postsynaptic differentiation. For example, similar presynaptic defects arise at the vertebrate neuromuscular synapse, when postsynaptic differentiation is prevented by disrupting the agrin/MuSK/rapsyn pathway.

WNTs have been implicated in synapse formation in the vertebrate CNS, as well (Salinas *et al.*, 2003). WNT7a is produced by cerebellar granule cells and influences the presynaptic morphology of mossy fiber axons, which ascend from the brainstem (Hall *et al.*, 2000). Mossy fiber synapses on granule cells typically form elaborate multisynaptic structures, called glomerular rosettes. The morphology of these rosettes is controlled by WNT7a signaling. The formation of glomerular rosettes is delayed in WNT7a knockout mice, and direct application of WNT7a to mossy fiber axons causes an accumulation of synapsin 1, an early molecular marker of synapse formation. The effects of WNT7a on terminal remodeling are blocked by a secreted Fz-related protein, which antagonizes WNT signaling, and are inhibited by lithium, which antagonizes GSK activity downstream of Fz receptor activation. Since WNT7a is made primarily by the postsynaptic cell, in this case, it appears to act as a retrograde factor for presynaptic differentiation.

Similar retrograde signaling by WNTs has also been observed in the spinal cord (Krylova *et al.*, 2002). In the lateral column of the ventral horn, neurotrophin 3 (NT3)-responsive primary muscle afferents form monosynaptic connections with spinal motor neurons. These motor neurons produce WNT3 during the development of these connections. Application of WNT3 to the NT3-responsive sensory axons decreases axonal growth, but increases axonal branching and growth cone size. These effects are blocked by secreted Fz-related protein and are mediated by GSK interaction with the microtubule cytoskeleton. Although these studies lack the *in vivo* genetic analysis performed for WNT7a in the cerebellum, together they represent a consistent picture of WNTs as retrograde signals for presynaptic

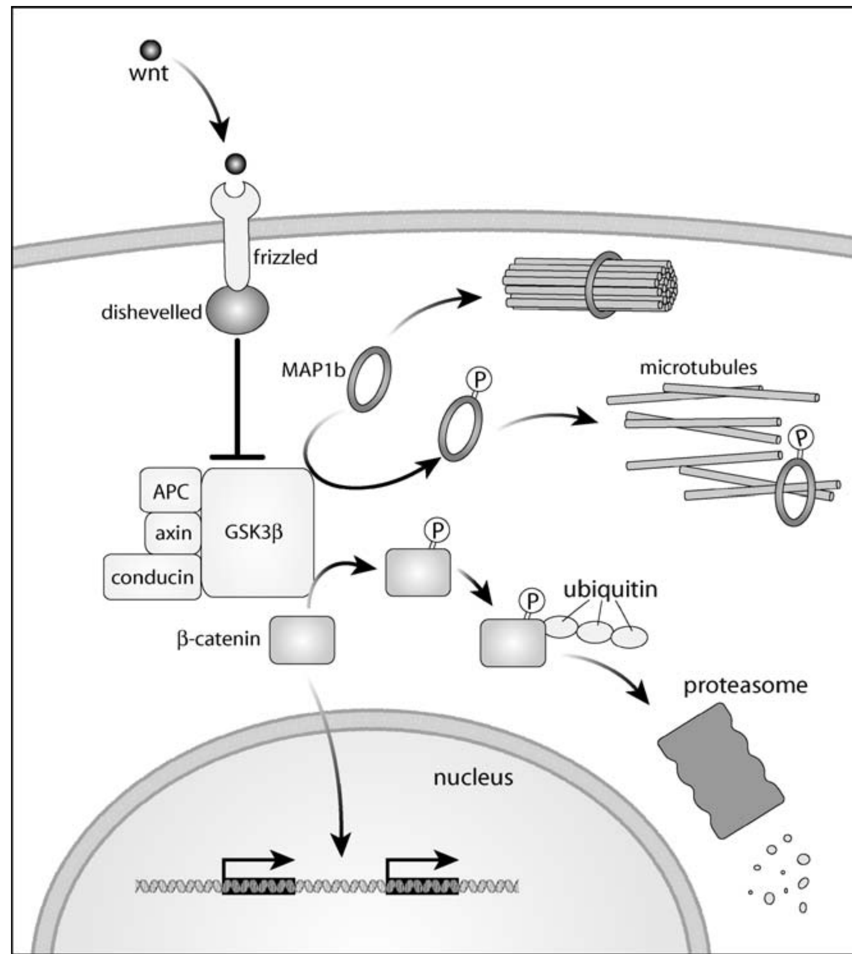


FIGURE 29. The wnt/frizzled pathway. WNT binding activates frizzled receptors, which leads to phosphorylation of dishevelled. Phosphorylated dishevelled inhibits GSK3β by promoting its association with APC. In the absence of WNT, active GSK3β phosphorylates MAP1b, which promotes dissociation of microtubule bundles. GSK3β also phosphorylates β-catenin, leading to its polyubiquitination and degradation. With WNT, phosphorylated dishevelled inhibits GSK3β, which stabilizes the microtubule cytoskeleton and allows levels of β-catenin to rise and regulate gene expression.

development in the vertebrate CNS. If WNTs prove to play roles in promoting presynaptic differentiation throughout the CNS, it will be important in determining how the specificity of synaptic connections is superimposed. The redundancy and complexity of the WNT/Fz signaling pathway represent an additional challenge.

Narp (Neuronal Activity-Regulated Pentraxin)

Narp was identified as an immediate early gene whose expression is induced by synaptic activity. Initially, activity-dependent regulation of Narp expression was taken as evidence that Narp functions after the initial steps in synaptogenesis, possibly to stabilize or refine initial connections (Tsui *et al*., 1996). More recent studies suggest that Narp may also play an important role at nascent synapses (O'Brien *et al*., 1999; Mi *et al*., 2002). Narp is selectively concentrated at glutamatergic synapses, which have been best studied in the hippocampus and spinal cord. Overexpression of Narp in cultured spinal neurons causes a substantial increase in the number of excitatory synapses present in the cultures. Narp co-aggregates with AMPA-type glutamate receptors after co-expression in non-neuronal cells, suggesting that it has a direct role in clustering glutamate receptors. However, Narp likely acts as a secreted factor to cluster receptors. For example, application of recombinant Narp to neuronal cultures causes cell-surface AMPA receptors to cluster. Thus, the activities of Narp on neuronal AMPA receptors are analogous to the activities of agrin on AChRs in cultured myotubes.

Several features of Narp deserve mention. First, the Narp polypeptide has homology to the pentraxin family of secreted proteins. Pentraxins form pentamers with a lectin-like three-dimensional structure. Lectins are plant proteins that bind with high avidity to carbohydrates. This and other biochemical features of Narp raise the interesting possibility that Narp acts as an extracellular bridge between carbohydrate moieties on neurotransmitter receptor or on receptor-associated proteins. Narp is secreted and could signal in anterograde fashion to promote

postsynaptic differentiation *in vivo*. Second, Narp is associated with glutamatergic synapses and is absent from inhibitory synapses. Narp may therefore promote the specificity of synaptic connections. Third, Narp acts at both spiny synapses in the hippocampus, and aspiny synapses in the spinal cord. The notion that one factor may influence two morphologically distinct classes of synapses is a refreshing bit of simplicity for the CNS. Fourth, as mentioned at the start, Narp expression is regulated by synaptic activity. This most interesting observation suggests Narp may play roles in maintaining or remodeling connections in the mature CNS.

Mechanisms of Postsynaptic Specialization

Effective neurotransmission at chemical synapses depends critically on the density of neurotransmitter receptors in the postsynaptic membrane. Mechanisms underlying the concentration of postsynaptic receptors were first identified at the NMJ. The importance of rapsyn to AChR clustering at the NMJ had seemed to argue that receptor-associated clustering agents would likely play a dominant role at all fast chemical synapses. This concept has received considerable support from subsequent studies, although it now appears that CNS synapses use different molecular components to similar ends, even at cholinergic synapses. Rapsyn, which clusters AChRs at the NMJ, is apparently a muscle-specific postsynaptic scaffolding component, as it is not significantly expressed in the CNS (even at cholinergic synapses). AChR clustering mechanisms at interneuronal cholinergic synapses have not been indentified. However, an analogous component, gephyrin, appears to cluster receptors at inhibitory synapses in the brain (Fig. 30; Kneussel and Betz, 2000).

Much like rapsyn, gephyrin is an intracellular protein that interacts directly and specifically with pentameric neurotransmitter receptors, in this case glycine and GABA receptors (Fig. 30). Gephyrin also anchors receptor complexes with intracellular cytoskeletal elements, much like rapsyn. However, gephyrin interacts with microtubules instead of the actin cytoskeleton. Genetic experiments support gephyrin's role in sustaining postsynaptic receptor clustering. Targeted genetic deletion of gephyrin by homologous recombination in mice results in a failure to cluster glycine and GABA receptors, and an absence of glycinergic and GABAergic synapses (Feng *et al*., 1998; Kneussel *et al*., 1999). Not surprisingly, the mutant mice cannot survive beyond birth. In humans, as well, autoimmune reactions directed against gephyrin cause "Stiff-Man Syndrome," a human disorder caused by a lack of inhibitory synaptic transmission in the CNS (Butler *et al*., 2000). These consistent series of observations were the first to definitively identify a specific receptor-clustering component in the CNS. Together, rapsyn and gephryn provide tangible evidence that tethering of postsynaptic receptors is a common mechanism of postsynaptic differentiation.

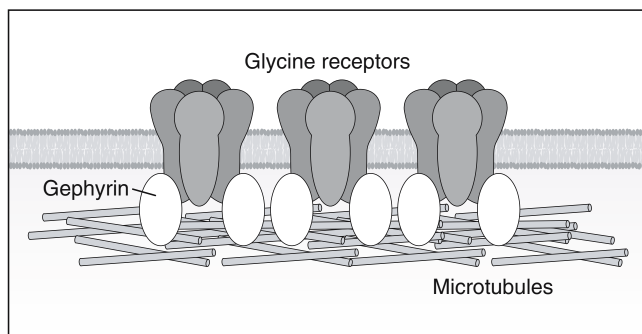


FIGURE 30. Glycine receptor clustering in the central nervous system is mediated by gephyrin. Gephyrin binds to the intracellular portion of the pentameric glycine receptors and also to the microtubule cytoskeleton. The role of gephyrin at inhibitory interneuronal synapses is analogous to the role of rapsyn at the neuromuscular junction.

Postsynaptic specializations in the CNS contain a large number of additional scaffolding proteins. One broad class is known by a particular element of protein tertiary structure involved in protein : protein interactions, the PDZ domain (reviewed in Nourry *et al*., 2003). PDZ domains were first identified in the tight junction protein ZO-1, the adherens junction protein Discs large (Dlg), and the 95 kDal postsynaptic density protein (PSD-95) concentrated at synaptic junctions in the vertebrate CNS (Kennedy, 1995). PDZ domains are present in all members of the PSD and SAP (*s*ynapse *a*ssociated *p*rotein) families, along with a catalytically inactive guanylate kinase homology domain. PDZ domains form hydrophobic pockets, which bind C-terminal amino acid motifs present on a number of transmembrane proteins. There is a loose consensus peptide sequence capable of interacting with PDZ domains. Most terminate with a valine residue, but differences at other positions promote preferential interactions with different PDZ domains.

The beauty of PDZ domain proteins is their modular structure. Multiple PDZ domains are typically present within a given polypeptide, in combinations with each other and additional protein interaction domains. PDZ domains are known to interact with glutamate receptors, potassium channels, and adhesion molecules, including neurexin and neuroligin discussed above. PSD-95, with three distinct PDZ domains, is able to interact with a neurotransmitter receptor, an ion channel, and a cell-adhesion molecule simultaneously. Thus, PDZ-proteins appear well-designed to link together multiple transmembrane and submembranous proteins. In this way, PDZ-proteins may serve to co-localize several functionally distinct membrane proteins that are fundamental to proper synaptic function. In this example, adhesion maintains proximity between pre- and postsynaptic elements, the neurotransmitter receptor responds to presynaptic exocytosis, and the ion channel propagates the depolarization into the neuron beyond. Although postsynaptic interactions involving PDZ-proteins are perhaps best described, PDZ domain proteins are also concentrated nerve terminals, where they may serve similar roles in linking presynaptic receptors, ion channels, and cell-adhesion molecules.

Glia-Derived Signals

Glial cells appear to be required for normal synaptogenesis. *In vivo*, synaptogenesis is concurrent with glial proliferation and

maturation. Where specific loss of glial cells has been induced, neurons are observed to withdraw their synaptic connections. *In vitro*, the number and strength of synaptic connections among, for example, cultured retinal ganglion cell neurons increases many-fold in the presence of astrocytes, or astrocyte-conditioned medium (Ullian *et al*., 2001; Slezak and Pfrieger, 2003). Despite these observations, a direct role for glial cells in promoting synapse formation is difficult to separate from their role in providing metabolic and trophic support to neurons. Glia absorb spent neurotransmitter and ions, which leach out of the synaptic cleft following transmission. They also provide trophic support to neurons. The ability of astrocytes to promote synapse formation *in vitro*, mentioned above, is associated with the ability of astrocytes to synthesize and supply cholesterol to the neurons (Mauch *et al*., 2001; Pfrieger, 2003). Neurons are especially rich in cholesterol, and cholesterol is especially concentrated in "rafts" in the plasma membrane, which are domains rich in signaling receptors. It is attractive to speculate that interneuronal signaling is regulated by a glia-derived supply of cholesterol, although there is little *in vivo* evidence to support this idea at present, and no clear evidence that cholesterol is present at limiting levels in normal neurons.

SYNAPTIC REMODELING

Throughout the nervous system, the initial pattern of innervation undergoes significant remodeling during postnatal development. This has been particularly well-studied in muscle, where serial images of single synapses can be obtained over the course of days, weeks, and months. The most significant changes in innervation result from modifications at the synaptic sites themselves (Fig. 31). Three main changes take place. First, a majority of the initial synaptic connections are eliminated. Second, the strength of individual connections is enhanced through structural changes that increase synaptic territory, in part to accommodate growth of the muscle fiber. Third, changes in the structure and geometry of the synapse are accompanied by upgrades to the molecular composition of the synapse. Some of these molecular alterations are known to require altered patterns of gene expression.

Synapse Elimination

In sharp contrast to the single axon that innervates each adult muscle fiber, at birth, each neonatal muscle fiber is

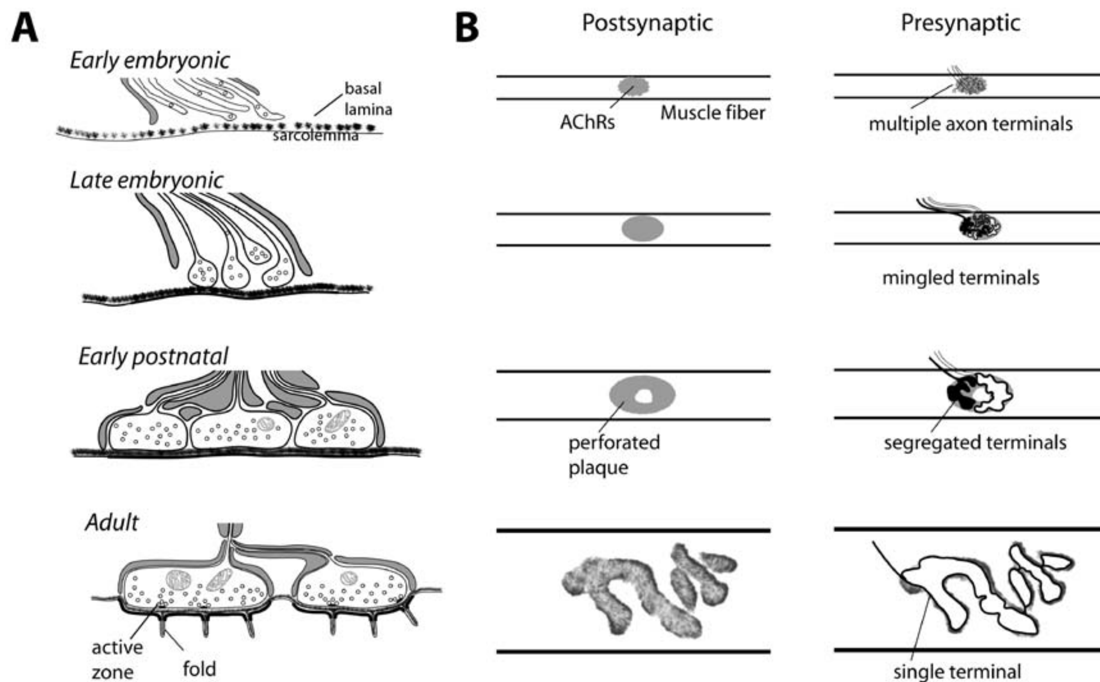


FIGURE 31. Development, maturation, and elimination of polyinnervation at neuromuscular synapses. Synapse formation in vertebrate muscles occurs in stages. (A) Embryonic myofibers are initially contacted by multiple motor axons, whose nerve terminals lack organized specializations and are loosely confederated within Schwann cell processes; postsynaptic sites have low concentrations of ACh receptors (AChRs) and a sparse basal lamina. Presynaptic terminals enlarge and concentrate synaptic vesicles, and postsynaptic membranes concentrate ACh receptors and specialized basal lamina components such as laminin β2 and ACh esterase, during embryonic and perinatal development. Secondary specializations, including active zones and folds, appear during postnatal maturation. (B) Pre- and postsynaptic development are spatially as well as temporally synchronized. Multiple axon terminals are initially co-mingled opposite a single AChR-rich plaque of postsynaptic membrane. As multiple inputs are eliminated through activity-dependent competition, surviving terminals become segregated, and holes appear in the postsynaptic plaque. Upon completion of synapse eliminated, the enlarged branches from a single nerve terminal innervate a matching series of postsynaptic gutters, which contain AChRs, a synapse-specific basal lamina, and secondary synaptic clefts.

innervated by multiple axons, typically from three to five (Fig. 31). Each of the axons innervating a single embryonic muscle fiber originates from a different motor neuron in the spinal cord. Nevertheless, their terminals interdigitate at a single, contiguous postsynaptic site on the muscle fiber (in twitch muscles; multiple postsynaptic sites appear on tonic muscle fibers). The apparent elevation in the number of synaptic connections present in neonatal muscle is real, compared to adult muscle. Neonates possess a mature number of spinal motor neurons, and a nearly complete number of muscle fibers. However, each spinal neuron has more intramuscular branches and innervates more muscle fibers in neonatal muscles than in adults. The large, overlapping motor units present at birth partly explain the exaggerated, uncoordinated movements of newborns.

Synapse elimination refers to the period during early postnatal development when all but one of the initial synaptic inputs to each fiber is disassembled. In a given muscle, most fibers become singly innervated within a few days of each other, although it may take several weeks to progress from the first to the final eliminated nerve terminal. Synapse elimination does not involve neuronal cell death, which is completed earlier. Instead, synapses are eliminated through the withdrawal of individual presynaptic terminals from the postsynaptic site (Bernstein and Lichtman, 1999). However, retraction of the preterminal axonal branch does bear similarity to the axonal atrophy that accompanies loss of trophic support, as if synapse elimination was a sort of subcellular, or subaxonal, demise.

The application of the term "synapse elimination" may at first seem confusing. Certainly, no neuron becomes targetless, and no muscle fiber becomes denervated. Rather, the number of neuron–muscle connections decreases by winnowing out the weakest connections from each hyperinnervated postsynaptic site. In addition, it is worth noting that the term "synapse elimination" does not describe the mechanism so much as the result. The term elimination might at first appear thoroughly myocentric, as it implies that the muscle fiber is the final arbiter in the decision of which of its inputs are rejected. As we shall see, current studies indicate that the muscle fiber does play a central role in *mediating* synapse elimination. However, the final outcome depends primarily on relative synaptic strengths and, therefore, relies as much on competitive interactions between the nerve terminals as on any controlling influence from the target itself. Synapse elimination must also be considered from the motor neuron's perspective, which selectively withdraws a majority of its embryonic nerve terminals, but necessarily succeeds in maintaining a substantial number as well.

The molecular mechanisms by which supernumerary nerve terminals are selectively eliminated from the muscle's postsynaptic site are not known. However, there are two known requirements to guide the ongoing search (Sanes and Lichtman, 1999). One requirement is postsynaptic activity in the muscle fiber. Simple paralysis of the muscle is in fact sufficient to prevent synapse elimination, strongly supporting the idea that retrograde factors play a role in eliminating connections. Polyinnervation persists on slowly contracting tonic muscles fibers, consistent with an absence of action potentials in these fibers.

A second requirement for normal synapse elimination is synaptic transmission. In particular, there must be *disparity* between both the strength and timing among the multiple axonal inputs whose terminals co-mingle on a given fiber. Synapse elimination in mice begins at neonatal ages, as motor neurons begin to lose gap-junctional coupling in the spinal cord, and electrical activity in the motor axons becomes temporally uncorrelated (Personius and Balice-Gordon, 2001). Complete neuromuscular blockade through pre- or postsynaptic mechanisms delays the elimination of polyinnervation; elimination proceeds when the block is released and neurotransmission is restored. Similarly, genetic perturbations that prevent release of neurotransmitter produce hyperinnervation, as in mice lacking the gene for choline acetyltransferase. In contrast, experimentally manipulating the levels of activity in a subset of axons supplying a muscle can accelerate the rate of elimination.

Recent genetic studies solidify support for the idea that it is the relative differences in synaptic activity that lead to the elimination of the weaker synapse. In one striking example, mice carrying a conditional mutation in choline acetyltransferase were used to eliminate ACh release from a subset of motor neurons, at neonatal ages (Buffelli *et al*., 2003). In competitions for synaptic territory between wild-type and ChAT-deficient axons, the "silent" axon always lost, despite equal conditions during development. Presumably, the absence of neurotransmitter did not affect axonal activity, rates of synaptic vesicle fusion, or access to other target- or Schwann cell-derived factors.

Similar results have come from studies of adult NMJs, in which neurotransmission through a small portion of the synapse was selectively blocked. Normally, the adult neuromuscular synapse is a model of stoic persistence, enduring with little structural change for the life of the animal (and hopefully a century in all of us). Nevertheless, these studies found that an adult NMJ will readily eliminate an entire lobe of the synapse, including pre- and postsynaptic elements, after focal blockade of neurotransmission in that lobe. Focal blockade was performed using a micropipette to flow a stream of irreversible AChR-antagonist (such as α-bungarotoxin) across one end of the target NMJ (Balice-Gordon and Lichtman, 1994). Repeated imaging of the same synapse over the ensuing days showed that the inactive portion of the synapse is always eliminated, without altering the structure of the active remainder of the junction. As noted above, blockade of the entire junctional area has the opposite effect, suppressing the elimination of differing inputs. Thus, by all tests, disparity in synaptic transmission is critical for synapse elimination.

The hypothesis that synapse elimination is driven by competitive interactions between neighboring synaptic inputs on a single target cell is now generally accepted. One axiom of this thesis is that competition is fueled by differences in the levels of synaptic activity, with active sites displacing inactive sites. A second axiom is that the target cell (here, the muscle fiber) mediates the competition between its synaptic inputs. Several major questions remain. What factor(s) serve as the molecular substrate of synaptic competition? How is synaptic activity coupled to the activity of putative maintenance/elimination factors? What postsynaptic mechanism(s) in the muscle interpret

different levels of synaptic activity between inputs and selectively eliminate the weakest? The answers to these questions are avidly sought, in part because they seem likely to apply to remodeling of synaptic connections throughout the nervous system, including the refinement of connections in the brain.

One possible mechanism for elimination at the NMJ is that motor nerve terminals compete for a retrograde trophic substance. Although none has been convincingly identified, it would presumably be available in limited amounts, and supplied in activity-dependent fashion by the muscle. In support of this idea, overexpression of glia-derived neurotrophic factor (GDNF) in the muscles of transgenic mice prevents synapse elimination and produces dramatic hyperinnervation (Nguyen *et al*., 1998). There is no direct evidence that GDNF or its like actually participate in regulating synapse elimination during normal development. Moreover, there is still no clear understanding of how a retrograde trophic factor could be differentially applied to terminals that vary only slightly in their temporal patterns of activity, or their physical location on the target. An alternative molecular mechanism posits that an alter ego to the retrograde trophic factor could provide the same competitive substrate. In this scenario, active terminals would be less susceptible to a toxic substance, such as a protease released by the muscle in response to synaptic activity. These putative activities have been dubbed "synaptotrophins" and "synaptotoxins," respectively (Sanes and Lichtman, 1999).

The mechanism by which muscles selectively couple differences in synaptic activity to differences in synaptic maintenance is an especially intriguing mystery. Careful observations of single NMJs show that the initial synaptic site is partially disassembled as synapse elimination proceeds. Repetitive observations of single NMJs during the period of synapse elimination show that nerve terminals undergoing elimination lose territory one branch at a time, starting in subregions of the synapse where they are especially underrepresented (Balice-Gordon and Lichtman, 1993; Balice-Gordon *et al*., 1993; Gan and Lichtman, 1998). Elimination of the terminal accelerates as the disparity in territory and efficacy increases (Colman *et al*., 1997; Kopp *et al*., 2000). Consistent with this accelerating disparity, synaptic sites that start out with evenly matched inputs are the last to complete the process of elimination. In addition, local disassembly of the postsynaptic apparatus beneath the losing terminal begins before the terminal completely withdraws. It seems likely that pre- and postsynaptic specializations that are destined for removal become molecularly distinguished from those that will be preserved. For example, activity in one region of the synapse could effectively "tag" adjacent regions, destabilizing them or marking them for disassembly. The nature of such a tag, the subsynaptic signals that would mediate differential tagging, and the mechanisms that could coordinate the removal of pre- and postsynaptic elements across the synaptic cleft remain speculative.

Structural Maturation

Paradoxically, synapse elimination occurs at the same time when the overall size and complexity of the NMJ is increasing (Fig. 30). First, the branches and varicosities of the nerve terminal thicken and fuse to form the mature terminal arbor. In parallel, the AChR-rich regions of the endplate are sculpted to precisely match the profile of the overlying nerve. One mechanism that likely helps maintain the precise colocalization of AChRs opposite the nerve terminal varicosities is incorporation of nerve-derived isoforms of agrin into the synaptic basal lamina. Agrin is required early in synaptic development to maintain AChR clusters at synaptic sites. At mature synapses, agrin is concentrated in the synaptic basal lamina immediately adjacent to high concentrations of AChRs in the postsynaptic membrane. Agrin is localized to the basal lamina of the primary synaptic cleft, between the nerve terminal and endplate, and immediately adjacent to the AChR-rich tops of the postsynaptic folds (Trinidad *et al*., 2000). Agrin is absent from basal lamina that lines postsynaptic folds, whose membranes lack AChRs. Agrin has been found to bind directly and avidly to laminin in the basal lamina. This interaction tethers agrin to its site of secretion, and thereby serves as a "blueprint" of the nerve terminal's dimensions for the developing AChR-rich endplate.

Second, the synapse begins to adopt a complex geometry. Regions of the synaptic area are subtracted, as competing nerve terminals are eliminated. However, the size of the surviving synaptic area increases in absolute size, as the muscle fiber grows in length and caliber, continuing into adulthood. Growth of the synaptic area occurs by intercalary addition since, like a child's hand, the overall geometry of each individual endplate retains its basic shape through development. Synaptic growth need not have been accomplished this way; for example, neuromuscular synapses in *Drosophila* larval muscles increase in size by budding new varicosities from the edge of previous ones.

Third, the muscle forms postsynaptic gutters beneath the terminal branches, and postsynaptic folds beneath the active zones. These modifications further enhance the strength of the synaptic connection by increasing the postsynaptic surface area, and by isolating sites of high-efficiency synaptic transmission. The mechanisms that promote the formation of gutters and folds are not understood. One possibility is that adhesive interactions between the nerve terminal and muscle endplate pull their shapes into conformity. Similar interactions between regions of the postsynaptic membrane could sustain the tightly formed folds, if not their formation. One possibility suggested by Jeff Lichtman is that folds reflect the constraints on the addition of membrane to a region of the muscle surface that is tightly bound to another surface, in this case the nerve terminal (Marques *et al*., 2000). If expansion of the postsynaptic surface is laterally constrained, it can only increase by puckering, like a bunched blanket. Consistent with this idea, postsynaptic folds are typically absent in mutant mice that lack synaptic isoforms of laminin, the basal lamina component which serves as a primary anchor to dystroglycan in the muscle membrane. According to this puckered-blanket model of the endplate, the postsynaptic membranes in mice lacking tight linkage to (and within) the synaptic basal lamina may be free to slide laterally as new postsynaptic membrane components are intercalated during growth of the muscle fiber.

Molecular Maturation

The molecular machinery that supports synaptic transmission and which maintains the integrity of the synaptic connection is modified as the synapse reaches maturity. One prominent modification is the substitution of the γ-subunit of the AChR present during embryonic development for the ε-subunit present in adult muscle. This switch in receptor composition is accompanied by changes in channel kinetics, which may be required for efficient signaling in large, adult muscle fibers (Missias *et al*., 1997). Voltage-dependent Na^{+} channels become concentrated in the depths of the postsynaptic folds at this time. Additional molecular changes increase the stability of the endplate receptors (Salpeter and Loring, 1985; Shyng *et al*., 1991), possibly through increased interactions with the submembranous cytoskeleton and the overlying synaptic basal lamina. For example, postnatal maturation of the dystrophin-associated protein complex, including dystrobrevin, is required to maintain the integrity of the AChR-rich postsynaptic domains (Grady *et al*., 1997, 1999, 2000; Adams *et al*., 2000). Similarly, the synaptic basal lamina undergoes a transition in composition during postnatal development; these changes are important to synaptic structure, as postnatal development of the synapse in the absence of basal lamina components, such as the laminin $\beta 2$, $\alpha 5$, and $\alpha 4$ chains, leads to major structural defects (Patton, 2003).

Synaptic maturation at the NMJ has functional counterparts in the maturation of synaptic connections in the brain, which are beyond the scope of this review (Cowan *et al*., 2001). During the postnatal development of the mammalian brain, for example, new synaptic territory is added as new neurons are added and dendritic fields enlarge. Broadly distributed projections are narrowed by increasing the number of synaptic connections in some areas and simultaneously eliminating connections in others. Finally, changes in synaptic strength are accompanied by alterations in the molecular composition of their pre- and postsynaptic elements. A common mechanism for change throughout the peripheral and central nervous systems is that structural changes are driven by relative levels of activity in neighboring synaptic connections. Activity-dependent changes in the synaptic architecture allow the initially crude, genetically determined pattern to adapt to best accommodate the host animal's interaction with the environment. Thus, remodeling likely represents an obligate solution to a fundamental problem in the development of any complex neural architecture: How to allocate synaptic connections in patterns which best fit the needs of each new member of the species.

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