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DEVELOPMENT OF THE VERTEBRATE NEUROMUSCULAR JUNCTION

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We dedicate this review to the memory of our friend, colleague, and collaborator, John Paul Merlie (1945–1995), honoring his many contributions to the understanding of synaptic development.

ABSTRACT

We describe the formation, maturation, elimination, maintenance, and regeneration of vertebrate neuromuscular junctions (NMJs), the best studied of all synapses. The NMJ forms in a series of steps that involve the exchange of signals among its three cellular components—nerve terminal, muscle fiber, and Schwann cell. Although essentially any motor axon can form NMJs with any muscle fiber, an additional set of cues biases synapse formation in favor of appropriate partners. The NMJ is functional at birth but undergoes numerous alterations postnatally. One step in maturation is the elimination of excess inputs, a competitive process in which the muscle is an intermediary. Once elimination is complete, the NMJ is maintained stably in a dynamic equilibrium that can be perturbed to initiate remodeling. NMJs regenerate following damage to nerve or muscle, but this process differs in fundamental ways from embryonic synaptogenesis. Finally, we consider the extent to which the NMJ is a suitable model for development of neuron-neuron synapses.

INTRODUCTION

The first rigorous demonstration of chemical synaptic transmission utilized the vertebrate skeletal neuromuscular junction (NMJ) (Dale et al 1936). Soon

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thereafter, this synapse was used by Bernard Katz and colleagues to show that the release of the neurotransmitter, acetylcholine, is quantal and vesicular (Katz 1966). Later, the first neurotransmitter receptor to be purified and then molecularly cloned was the nicotinic acetylcholine receptor (AChR) (Duclert & Changeux 1995). All of these studies, combined with the experimental accessibility of the NMJ, have made it the leading preparation for analysis of synaptic development. Although its hegemony is now threatened by growing knowledge about synaptogenesis in *Drosophila* (Keshishian et al 1996) and the mammalian hippocampus (see, for example, Rao et al 1998), the vertebrate NMJ is still the synapse about whose formation we know the most.

Studies of neuromuscular development have spanned a century, with progress determined in large part by the tools available. At the turn of the century, Fernando Tello, a student of Santiago Ramon y Cajal, used the light microscopic methods of his mentor to elucidate some of the main features of neuromuscular synaptogenesis and regeneration (Cajal 1928). Few fundamental advances were made for several decades thereafter. During the 1950s, however, the then-new techniques of intracellular recording and electron microscopy were applied to the adult NMJ, resulting in a sophisticated view of its structure and function that led to reinvestigation of its development. These descriptive studies were followed in turn by experimental analyses during the 1970s, leading to the realization that synapse formation involves elaborate signaling between nerve and muscle (Dennis 1981). Later, the advent of hybridoma and molecular biological techniques permitted isolation of several candidate signaling molecules and demonstration of their bioactivities in vitro (Hall & Sanes 1993). During the present decade, technical advances in imaging and molecular genetics have permitted critical tests in vivo of specific hypotheses derived from earlier work. Accordingly, our emphasis in this review is on mechanisms that regulate the formation, maturation, maintenance, and regeneration of NMJs in vivo. In an effort to avoid losing sight of the forest as we catalogue the trees, we have summarized the main points of each section in a figure.

THE CAST OF CHARACTERS (FIGURE 1)

The NMJ comprises portions of three cells—motor neuron, muscle fiber, and Schwann cell (reviewed in Couteaux 1973, Ogata 1988, Engel 1994). The cardinal feature of synaptic structure is that synaptic portions of all three cells are highly specialized, containing high concentrations of organelles and molecules found at low concentrations extrasynaptically.

The motor nerve terminal is specialized for neurotransmitter release. It bears large numbers of 50-nm-diameter synaptic vesicles that contain the neurotransmitter, acetylcholine, as well as numerous mitochondria, which provide the energy for synthesis and release of transmitter. The terminal is polarized, with

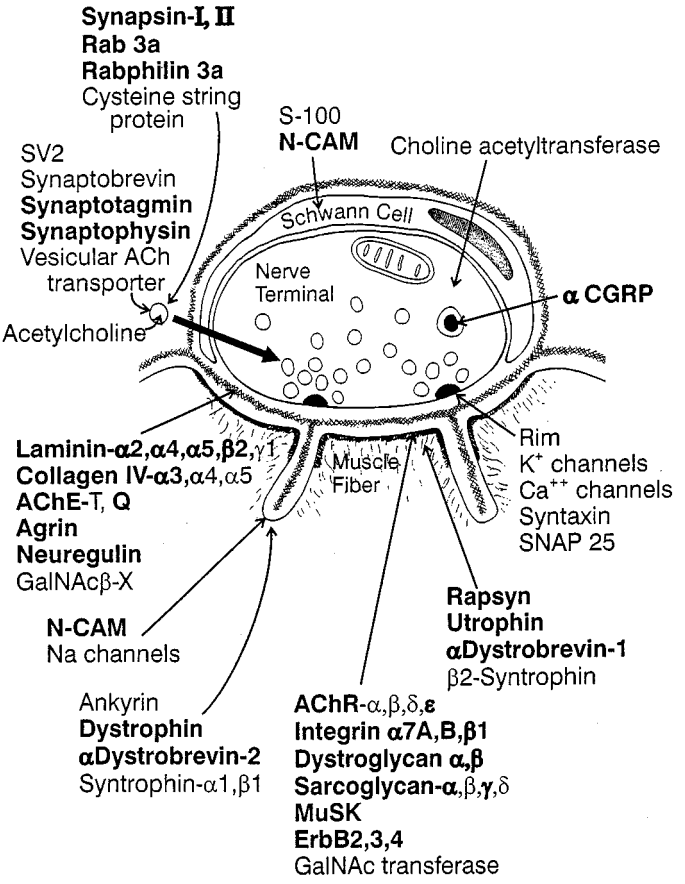

Figure 1 Structure and molecular architecture of the neuromuscular junction. The nerve terminal occupies a shallow gutter in the muscle fiber and is capped by processes of Schwann cells. Active zones in the nerve terminal directly appose junctional folds in the postsynaptic membrane. Some of the proteins concentrated at the synapse are shown, with their subcellular localizations indicated by arrows. Those for which knockout mice have been generated are indicated in boldface. See text (Hall & Sanes 1993, Grinnell 1995) for primary references and names of additional components.

most of the vesicles clustered in the half-terminal that faces the muscle fiber, and most of its mitochondria in the half-terminal beneath the Schwann cell. Many of the vesicles are further focused at dense patches on the presynaptic membrane, called active zones, at which vesicles fuse with the membrane to release their contents into the synaptic cleft. Preterminal portions of the motor axon contain fewer vesicles and mitochondria than terminals, and no active zones. Conversely, terminals contain few microtubules and neurofilaments,

which are abundant preterminally (Yee et al 1988). The best-studied molecules of the nerve terminal are the proteins of the synaptic vesicle (reviewed in Calakos & Scheller 1996, Sudhof 1995), as well as the neurotransmitter, its synthetic enzyme (choline acetyltransferase), and the vesicular acetylcholine transporter. Few components of the active zone have been identified, but this structure is associated with voltage-dependent potassium and calcium channels, whose localization to transmitter release sites maximizes the efficacy of calcium-dependent secretion (Robitaille et al 1993, Sugiura et al 1995, Day et al 1997).

The postsynaptic membrane is specialized to respond rapidly and reliably to neurotransmitter released from the overlying nerve terminal. It bears an extremely high concentration of AChRs ($>10{,}000/\mu m^2$) (reviewed in Salpeter & Loring 1985). Several others signaling molecules are also concentrated in the membrane (Moscoso et al 1995a, Valenzuela et al 1995, Zhu et al 1995, PT Martin et al 1996, see Hall & Sanes 1993 for earlier references). Associated with the cytoplasmic segments of the AChRs is an elaborate cytoskeletal apparatus described below, a principal function of which is to generate and maintain the high synaptic AChR density in the face of a sharp decline to the extrasynaptic AChR density of $\sim 10/\mu m^2$ (Salpeter et al 1988).

The postsynaptic membrane of the muscle fiber is depressed into shallow gutters beneath the nerve terminal, and then further invaginated into $\sim$1-μm-deep junctional folds that open directly opposite active zones. AChRs are concentrated at the crests and partway down the sides of the folds, whereas sodium channels and the neural cell adhesion molecule (N-CAM) are concentrated in the depths of folds (Covault & Sanes 1986, Flucher & Daniels 1989), an arrangement that may enhance the efficacy of synaptic transmission (Wood & Slater 1997). The cytosketon of the folds is also heterogeneous: Rapsyn, utrophin, and α-dystrobrevin-1 are colocalized with AChRs at the tops of folds, while ankyrin, α-dystrobrevin-2, and dystrophin are concentrated at the bottoms of folds (Sealock et al 1984, Covault & Sanes 1986, Flucher & Daniels 1989, Wood & Slater 1998, Peters et al 1998). Cytoskeletal elements are likely to be involved not only in generating the folds but also in maintaining the different domains within them.

Schwann-cell processes cap the nerve terminal, insulating it from the environment and perhaps providing it with trophic sustenance. In contrast, Schwann cells in contact with preterminal portions of the axon form myelin sheaths. These two types of Schwann cells arise from the same progenitors, but differ structurally and express different genes—for example, myelin-forming Schwann cells are rich in myelin basic protein, myelin-associated glycoprotein, and P_0, but poor in N-CAM and S-100, whereas the opposite is true for terminal Schwann cells (Mirsky & Jessen 1996).

Finally, a basal lamina ensheaths each muscle fiber, passes through the synaptic cleft, and extends into the junctional folds. The major components of muscle basal lamina are similar to those of basal laminae throughout the body—collagen IV, laminin, entactin, and heparan sulfate proteoglycans. However, synaptic and extrasynaptic portions of the basal lamina differ in their isoform composition. Synaptic basal lamina also contains a collagen-tailed form of acetylcholinesterase (Krejci et al 1997), a set of glycoconjugates (Scott et al 1988), and two signaling molecules, agrin and neuregulin, which are discussed below.

APPROACH AND CONTACT (FIGURE 2)

Origin of Cells

All three cells of the NMJ travel long distances to meet at the synapse (Figure 2*a*). Muscle fibers are derived from mesodermal cells that acquire a myogenic identity in the dermatomyotomal portion of the somites (reviewed in Brand-Saberi et al 1996). Committed myogenic cells then migrate to sites where muscles will form. There they divide, and their postmitotic progeny differentiate into myoblasts. The myoblasts align into "straps," then fuse to form myotubes (Figure 2*b*). Genes that encode many contractile and synaptic proteins are activated on fusion (reviewed in Ontell & Ontell 1995), but maturation of the myotube is a protracted process. By convention, the movement of myonuclei from the core of the cylinder to its periphery is taken as a cardinal sign of maturity; multinucleated muscle cells are called myotubes when they are centrally nucleated and muscle fibers once they become peripherally nucleated.

Motoneurons arise in the ventral portion of the neural tube from multipotential progenitors that also give rise to interneurons and glial cells (Leber et al 1990). Motor axons exit the central nervous system through ventral roots or cranial nerves, then run long distances through peripheral nerves to muscles. Motor axons seldom branch en route, and therefore innervate only a single muscle. They do branch numerous times intramuscularly, however, to innervate tens to hundreds of muscle fibers. The combination of a motoneuron plus all of the muscle fibers it innervates is called a motor unit.

Schwann cells, the glia of the peripheral nervous system, are derivatives of the neural crest, which arises from the dorsal margin of the neural tube (Figure 2*a*). Both Schwann cells and motor axons traverse the rostral halves of the somites, from which they derive their segmental arrangement. It is probably within or near the somites that Schwann cells become associated with motor axons. Thereafter, Schwann cells follow motor axons through the periphery and into muscles. During this journey, the axons provide both migratory guidance and mitogenic stimulation for the Schwann cells (reviewed in Mirsky & Jessen

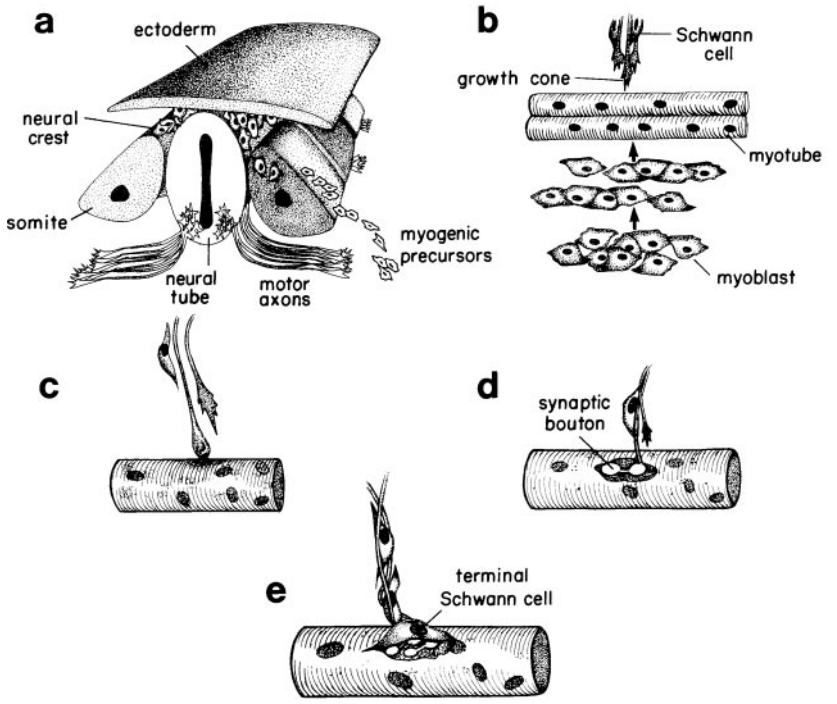

Figure 2 Early steps in the formation of the neuromuscular junction. (*a*) Origin of the cells that comprise the NMJ. Myoblasts arise from the somite, motor axons from somata in the neural tube, and Schwann cells from the neural crest. All three cells travel long distances to meet at the NMJ. (*b*) Once they reach sites of muscles, myoblasts fuse to form myotubes. Just as or after they form, they are approached by motor axons, followed closely by Schwann cells. (*c*) Initial contacts are unspecialized yet capable of rudimentary transmission. (*d*) As development proceeds, growth cones differentiate into nerve terminals, and AChRs accumulate in the postsynaptic membrane. (*e*) By birth, the NMJ is fully functional and multiply innervated.

1996). There have been suggestions that Schwann cells lead rather than follow motor axons and therefore play an active role in guiding axons. At present, the preponderance of evidence is that axons lead, with Schwann cells following closely behind (Riethmacher et al 1997); in regeneration, however, the order is reversed (see below).

Two features of early cellular differentiation are especially relevant to the subsequent processes of synaptogenesis. First, motoneurons, muscle cells, and Schwann cells all acquire their identities before synapses form, and express genes encoding synaptic components when cultured in isolation. Thus, intercellular interactions that regulate synaptic development are more organizational

than strictly inductive in the classical embryological sense. Second, although all motoneurons share numerous features, as do all muscle fibers, both cell types display considerable molecular heterogeneity prior to the onset of synaptogenesis (reviewed in Tanabe & Jessell 1996, Donoghue & Sanes 1994). These differences are likely to be critical not for the formation of NMJs per se, but for preferential formation of NMJs between appropriate partners and for intermuscular differences in NMJ structure.

Nerve-Muscle Contact

Motor axons reach target muscles as myoblasts are fusing to form myotubes. Once the motor axon's growth cone contacts a newly formed myotube, synaptic transmission commences quickly. Initially, however, the efficacy of transmission is extremely low, reflecting the absence of both pre- and postsynaptic specializations. Over a period of about a week, a fully functional (albeit immature) synapse forms in which both nerve and muscle are greatly transformed (Figure 2*c–e*). (All intervals and stages refer to mice unless otherwise specified.)

In adult muscles, synapses are clustered in a central end-plate band, giving the impression that the midpoints of muscle fibers are especially susceptible to innervation. In fact, it is more likely that axons contact myotubes near their sites of entry into the muscle, and form synapses at sites of contact. Axons fasciculate to form a single intramuscular nerve, and myotubes grow symmetrically at their ends (Zhang & McLennan 1995). These patterns suggest that the sterotyped positions of synapses on muscle fibers result from coordinated nerve and muscle growth rather than from the existence of preordained synaptic sites (Bennett & Pettigrew 1976). Direct observation of synapse formation in nerve-muscle cocultures supports this view: Even though some high-density clusters of AChRs form spontaneously in uninnervated myotubes, axons do not seek them out but rather organize new clusters at initially unspecialized sites (Anderson & Cohen 1977, Frank & Fischbach 1979).

POSTSYNAPTIC DIFFERENTIATION (FIGURE 3)

AChR subunit genes are expressed at low levels in myoblasts, then upregulated during fusion, as part of the myogenic program that also leads to formation of the contractile apparatus. The AChR subunits are translated, assembled, and inserted in the plasma membrane, where they soon reach a uniform density of $\sim 1000/\mu m^2$ (Bevan & Steinbach 1977, Merlie 1984). In mature muscle, in contrast, the density of AChRs reaches $>10{,}000/\mu m^2$ synaptically and falls to $\sim 10/\mu m^2$ within a few 10s of microns from the nerve terminal's edge (see above). Three distinct processes contribute to this redistribution: clustering of diffusely distributed AChRs in the postsynaptic membrane, transcriptional

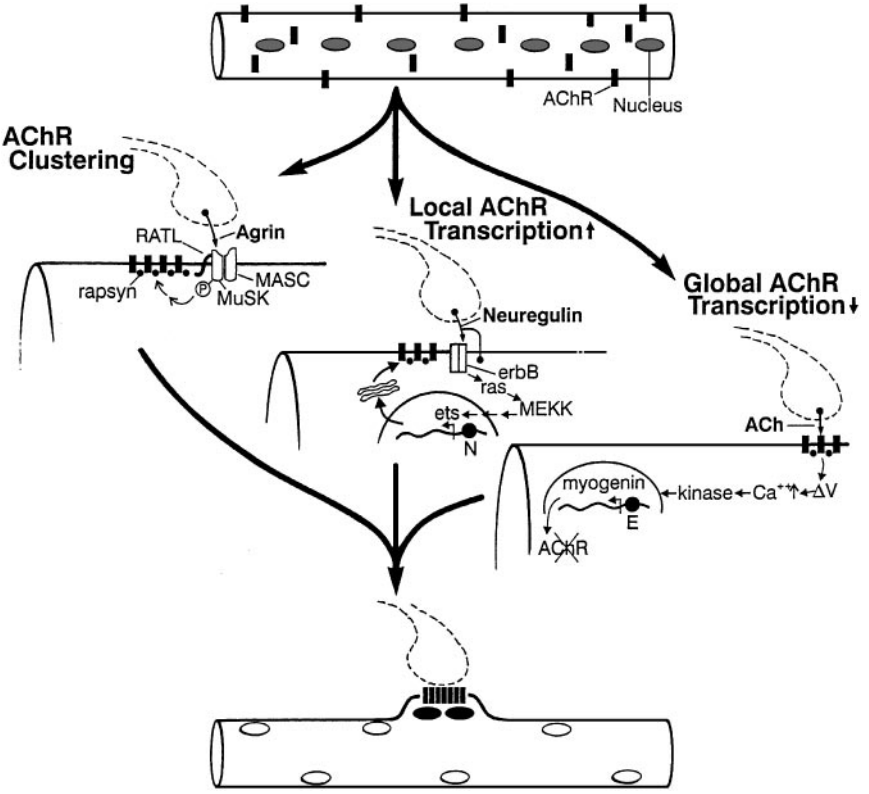

Figure 3 Accumulation of AChRs in the postsynaptic membrane. Nuclei throughout newly formed myotubes express AChR subunit genes, and AChRs are diffusely distributed on the myotube surface. The nerve then sends three signals to the muscle that affect AChR distribution. One is agrin, which interacts with MuSK to organize rapsyn-mediated AChR clustering. The second is neuregulin, which interacts with erbB kinases to induce selective expression of AChR subunit genes by synaptic nuclei. This effect is mediated in part by binding of ets family transcription factors to N-box sequences in AChR gene promoters. The third is acetylcholine, which activates AChRs to generate a voltage- and calcium-dependent signal that represses AChR subunit gene expression in extrasynaptic nuclei. This effect is mediated in part by binding of myoD-family transcription factors to E-box sequences in AChR gene promoters. Together, these signals lead to selective synthesis of AChRs in synaptic areas and precise accumulation of AChRs in the postsynaptic membrane.

activation of AChR subunit genes in subsynaptic nuclei, and transcriptional repression of AChR subunit genes in nonsynaptic myonuclei. The nerve controls all three processes, but in different ways.

Clustering

Following the demonstration that neurites organize AChR clusters at sites of nerve-muscle contact (Anderson & Cohen 1977, Frank & Fischbach 1979), several groups used cultured myotubes to seek clustering agents. Typically, a fluorescent derivative of α-bungarotoxin, a quasi-irreversible ligand, was used to assay AChR distribution. In some cases, labeled α-bungarotoxin was applied before the test substance, so that preexisting AChRs would be labeled but AChRs induced by the treatment would not. Proteins that are active in this assay include fibroblast growth factor (Peng et al 1991), HB-GAM/pleitrophin (Peng et al 1995, Rauvala & Peng 1997), laminin (Sugiyama et al 1997, Montanaro et al 1998), midkine (Zhou et al 1997), and agrin. Of these, only agrin has so far been implicated in synaptogenesis in vivo.

Agrin was isolated by McMahan and colleagues from Torpedo electric organ, a plentiful source of NMJ-like synaptic components (Godfrey et al 1984, Nitkin et al 1987), then molecularly cloned from mammals and birds (Rupp et al 1991, Ruegg et al 1992). Native agrin is a heparan sulfate proteoglycan of $\sim$400 kDa (Tsen et al 1995, Denzer et al 1995). Agrin is synthesized by motoneurons, transported down motor axons, released from motor nerve terminals, and incorporated into the basal lamina of the synaptic cleft (McMahan 1990, Reist et al 1992, Cohen & Godfrey 1992). Based on its patterns of expression and bioactivity, McMahan (1990) proposed that agrin is a critical nerve-derived organizer of postsynaptic differentiation.

Recent gain- and loss-of-function studies support this agrin hypothesis. To overexpress agrin in the absence of the nerve, expression vectors were injected into muscles or microinjected into denervated muscle fibers (Jones et al 1997, Meier et al 1997, Rimer et al 1997, Cohen et al 1997). In both cases, patches of recombinant agrin formed on aneural surfaces of transfected muscle fibers or their neighbors, and remarkably complete postsynaptic apparati formed in precise apposition to the deposits of agrin. Thus, agrin can account for much of the synaptic organizing ability of the nerve. Conversely, postsynaptic differentiation was profoundly impaired in agrin-deficient mutant (knockout) mice: Overall levels of AChR were normal, but few nerve-muscle contacts were accompanied by detectable AChR clusters or other postsynaptic specializations (Gautam et al 1996, Burgess et al 1998). In addition, motor axons failed to arborize properly on agrin$^{-/-}$ myotubes. Thus, agrin is necessary as well as sufficient for postsynaptic differentiation.

Although agrin is confined to synaptic sites in adult muscle fibers, it is synthesized by muscle as well as nerve and is present throughout the basal lamina of developing mytotubes (Fallon & Gelfman 1989, Bowe & Fallon 1995). Initially, this distribution seemed inconsistent with the hypothesis that agrin serves as a localized, nerve-derived signal. A likely resolution to this paradox came with the discoveries that the agrin gene is subject to alternative splicing, that inclusion of exons at a carboxy-terminal site (called B in birds and z in mammals) greatly potentiates and may be required for the AChR clustering activity of agrin, and that neurons but not muscles express the B+ or z+ agrin isoforms (Ruegg et al 1992; Ferns et al 1992, 1993; Hoch et al 1993; Gesemann et al 1995). It remained possible, however, that muscle as well as nerve agrin affected synaptic differentiation. This issue has now been addressed in two ways (Burgess et al 1998). First, agrin$^{-/-}$ muscles were transplanted to wild-type hosts, where they were reinnervated by agrin-expressing axons. Synapses in these surgical chimeras displayed neither pre- nor postsynaptic defects. Second, a novel allele was generated in which the z exons were deleted with only minor effects on the levels of z-muscle agrin. Both pre- and postsynaptic defects in these second-generation mutants were indistinguishable from those in a null allele. Thus, it is nerve-derived z agrin that is essential for synaptic development.

Agrin Signaling

Several molecules on the myotube surface are capable of interacting with agrin. These include dystroglycan, glycoconjugates, integrins, HB-GAM/pleitrophin, heparan sulfate proteoglycans, N-CAM, and laminins (Bowe et al 1994; Campanelli et al 1994, 1996; Gee et al 1994; Sugiyama et al 1994; Martin & Sanes 1995, 1997; Mook-Jung & Gordon 1995; Storms et al 1996; Daggett et al 1996; Gesemann et al 1996; O'Toole et al 1996; Hopf & Hoch 1996; Denzer et al 1997). Several of these have been implicated in AChR clustering based on studies in culture myotubes, but none has been shown to be critical for postsynaptic differentiation in vivo (reviewed in Sanes et al 1998). In contrast, a transmembrane protein tyrosine kinase, MuSK, originally identified in an unrelated search for signaling molecules in muscle, has now emerged as the best candidate agrin receptor, even though it apparently does not bind agrin.

MuSK is selectively expressed by skeletal muscle, where it is co-localized with AChRs in the postsynaptic membrane (Valenzuela et al 1995, Ganju et al 1995). It is homologous to an orphan tyrosine kinase previously cloned from Torpedo electric organ (Jennings et al 1993). Unexpectedly, MuSK$^{-/-}$ knockout mice displayed neuromuscular defects similar to—and if anything, more severe than—those of the agrin$^{-/-}$ mutants described above: Muscles bore some AChR clusters in the latter but none in the former, even though both synthesized

normal levels of AChRs (DeChiara et al 1996). This phenotype immediately elevated MuSK to the status of leading-candidate agrin receptor.

Several additional results support this view (Glass et al 1996, 1997; Meier et al 1996; Hopf & Hoch 1998a,b; H Zhou & JR Sanes, in preparation; M Gautam et al, submitted). First, myotubes cultured from MuSK$^{-/-}$ mice fail to form AChR clusters, either spontaneously or in response to agrin, and this defect can be rescued by reintroduction of MuSK. Second, expression of a dominant-negative mutant MuSK in wild-type cells inhibits agrin's ability to induce AChR clustering. Third, application of agrin to myotubes leads to rapid phosphorylation of MuSK. Fourth, application of chemical cross-linkers to muscle cells leads to covalent attachment of agrin to MuSK. Finally, only isoforms and fragments of agrin that stimulate AChR clustering lead to MuSK activation or can be cross-linked to MuSK. The main difficulty at present is that it has been impossible to demonstrate binding of purified agrin either to purified MuSK or to MuSK expressed in nonmuscle cells. This suggests that MuSK is one subunit of a multisubunit receptor, with other subunits being either necessary or solely responsible for agrin binding. The specificities of the agrin-binding proteins listed above make them unlikely candidates for coreceptor, and efforts are under way to purify an additional subunit, tentatively dubbed muscle-associated specificity component, or MASC (Glass et al 1996).

Downstream of MuSK, a critical effector of AChR clustering is a 43-kDa cytoplasmic protein called rapsyn, originally cloned by Merlie and colleagues (Frail et al 1987). AChRs and rapsyn are precisely colocalized at NMJs as soon as clusters form, are present at adult NMJs in a 1:1 stoichiometry, and may bind directly to AChRs (Burden et al 1983, Sealock et al 1984, Noakes et al 1993). Moreover, AChRs are diffusely distributed when expressed in heterologous cells, but form high-density clusters when coexpressed with rapsyn (Froehner et al 1990, Phillips et al 1991); rapsyn bears separable domains responsible for association with the membrane, multimerization, and interaction with AChRs (Ramaroa & Cohen 1998). Conversely, no AChR clusters form in muscles of rapsyn-deficient mutant mice, or in myotubes cultured from the mutants, even following treatment with agrin (Gautam et al 1995). Thus rapsyn is necessary for AChR clustering.

Although genetic studies place agrin, MuSK, and rapsyn in a pathway, they leave open questions of what steps intervene between agrin and MuSK and between MuSK and rapsyn. Recent results provide initial insights into these issues. First, MuSK is clustered at synaptic sites in rapsyn$^{-/-}$ mice, even though AChRs and numerous other synaptic proteins are diffusely distributed in these mutants (Gautam et al 1995, Moscoso et al 1995a, Apel et al 1997). Thus, MuSK is a critical component of a primary synaptic scaffold, and rapsyn recruits other synaptic components to that scaffold. Agrin may both cluster and

activate MuSK, but different domains of agrin are responsible for these two activities (MA Ruegg, submitted). Second, whereas the sole role of the extracellular domain in most receptor tyrosine kinases is to allow ligand to activate the cytoplasmic domain, the extracellular domain of MuSK is crucial for forming the primary scaffold and promoting MuSK-rapsyn interactions (Gillespie et al 1996, Apel et al 1997). This arrangement provides a means whereby MuSK can not only induce formation of postsynaptic specializations but also localize those specializations precisely to synaptic sites. Third, the intracellular substrate of MuSK is likely to be another kinase, possibly a member of the src family (Fuhrer & Hall 1996, Glass et al 1997, Fuhrer et al 1997). This kinase, in turn, phosphorylates AChRs and other still-unidentified synaptic components required to trigger clustering (Wallace et al 1991, Wallace 1995, Ferns et al 1996), possibly by a calcium-dependent step (Megeath & Fallon 1998). Finally, laminin-1 and a plant lectin can induce AChR clustering in MuSK-deficient but not in rapsyn-deficient myotubes (Sugiyama et al 1997; Montanaro et al 1998; Gautam et al, submitted). The physiological significance of these stimuli is unknown, but their activities demonstrate that MuSK is not a structural component of AChR clusters, and that MuSK-independent pathways of clustering exist, but that rapsyn is required for clustering per se.

Synapse-Specific Transcription

In vivo, the few myonuclei directly beneath the postsynaptic membrane transcribe AChR subunit genes at far higher rates than the hundreds of nonsynaptic nuclei that share the same cytoplasm (Merlie & Sanes 1985, Goldman & Staple 1989, Klarsfeld et al 1991, Sanes et al 1991, Simon et al 1992, reviewed in Duclert & Changeux 1995). In vitro, innervation of myotubes or application of neural extracts leads to increased synthesis of AChRs (Jessell et al 1979). Together, these results suggest that the nerve provides a localized signal that stimulates AChR gene expression in subsynaptic nuclei. Localized transcriptional activation, in turn, results in localized synthesis of AChRs, and thereby contributes to the synaptic accumulation of AChRs. Two molecules have been proposed as nerve-derived inducers of synapse-specific transcription: calcitonin gene-related peptide (CGRP) and neuregulin.

CGRP is produced by alternative splicing from the gene originally shown to encode the hormone calcitonin. Whereas calcitonin is synthesized predominantly in the thyroid gland, CGRP is present in a variety of central and peripheral neurons. In motoneurons, it is packaged into dense core vesicles, transported to motor nerve terminals, and released on stimulation (Uchida et al 1990, Changeux et al 1992). Application of CGRP to cultured myotubes stimulates AChR synthesis (New & Mudge 1986; Fontaine et al 1986, 1987).

Fischbach and colleagues isolated an acetylcholine receptor-inducing activity (ARIA) from brain, based on its ability to stimulate AChR accumulation in cultured myotubes (Usdin & Fischbach 1986). The purified protein of ~42 kD had no apparent AChR clustering activity, but did increase AChR subunit mRNA levels, suggesting a transcriptional effect (Martinou et al 1991). Molecular cloning (Falls et al 1993) revealed that ARIA was one of many alternatively spliced products of a gene now called neuregulin; other products of the neuregulin gene had been isolated as ligands of the neu/erbB protooncogene (heregulin and neu differentiation factor) and as a glial growth factor (reviewed by Fischbach & Rosen 1997). Neuregulin, like CGRP, is synthesized by many cell types, including motoneurons. Moreover, it is transported down motor axons, and becomes incorporated into synaptic basal lamina, probably by binding to heparan sulfate proteoglycans (Goodearl et al 1995, Loeb & Fischbach 1995).

Both neuregulin and CGRP are reasonable candidate inducers of AChRs. However, loss-of-function studies support the candidacy of neuregulin but not that of CGRP. Synaptic development was not detectably impaired in mutant mice lacking αCGRP (JT Lu et al, submitted). As to neuregulin, homozygous neuregulin-deficient mutant embryos die of cardiac defects before NMJs form (Meyer & Birchmeier 1995), so simple genetic tests of its role in synaptogenesis are currently infeasible. In heterozgotes, however, neuregulin mRNA levels are decreased by 50% relative to controls, and this deficiency results in 50% decreases in the level of synapse-associated AChR mRNA and in the density of synaptic AChRs (Sandrock et al 1997).

Although this result indicates that neuregulin regulates AChR synthesis, its interpretation is complicated in two respects. First, neuregulins account for a major fraction of the mitogenic activity that motor axons provide to Schwann cells (Dong et al 1995, Trachtenberg & Thompson 1996, Grinspan et al 1996), and Schwann cells, in turn, are required for complete differentiation of the nerve terminal (see below). Thus, neuregulin could affect postsynaptic properties both directly and indirectly. Second, muscles as well as motoneurons synthesize neuregulin (Moscoso et al 1995a), raising the possibility that it could act downstream of agrin. Indeed, neuregulin and neuregulin receptors are concentrated at postsynaptic specializations induced by agrin in aneural muscle (Meier et al 1997, Rimer et al 1997). Moreover, agrin can induce AChR gene expression in cultured myotubes (Jones et al 1996), and this effect is blocked by antagonists of neuregulin receptors (Meier et al 1998). Thus, muscle-derived neuregulins could serve as second messengers to agrin. In summary, although neuregulin is likely to be an important regulator of postsynaptic differentiation, its relevant sources and direct targets remain uncertain.

Neuregulin Signaling

The first neuregulins were isolated as ligands of the neu protooncogene, an epidermal growth factor–related (erbB) receptor tyrosine kinase. The identification of ARIA as a neuregulin therefore suggested that its receptors were erbB kinases. In fact, three of the four known erbB genes (erbB2, 3, and 4; erbB1 is the EGF receptor) are concentrated in the postsynaptic membrane, and application of neuregulin to muscle cells rapidly activates erbB kinases (Jo et al 1995, Chu et al 1995, Moscoso et al 1995a, Zhu et al 1995, Altiok et al 1995). Unfortunately, mutant mice lacking erbB2, 3, or 4 all die at early embryonic stages (Lemke 1996, Riethmacher et al 1997), and it remains unclear which erbB isoforms mediate neuregulin's effects on muscle cells.

Based on the assumption that neuregulins act through erbB receptors, several groups have begun to elucidate the steps between activation of the kinase and transcriptional activation of the AChR genes. In cultured myotubes, erbB kinases signal through a generic cascade of kinases, including ras, raf, erk, and phosphatidylinositol-3-kinase (Tansey et al 1996, Si et al 1996, Altiok et al 1997). These kinases, in turn, activate AChR gene transcription. Neuregulin-responsive elements have been defined in regulatory regions of several AChR subunit genes, and in at least some cases, these sequences correspond to regions that confer synapse-specific expression on reporters in vivo (Gundersen et al 1993, Tang et al 1994, Koike et al 1995, Duclert et al 1996, Si et al 1997, Sapru et al 1998). A crucial element, called an N-box by Changeux and colleagues (Koike et al 1995), corresponds to the consensus binding motif for transcription factors of the ets family, which are known to mediate effects of some ras-dependent signaling cascades in nonneural tissues. Indeed, several members of this family are expressed in muscle, and two of them, GABPα and GABPβ, bind to the AChR genes (Sapru et al 1998, Schaeffer et al 1998, Fromm & Burden 1998). Thus, signaling pathways that are used by numerous receptor tyrosine kinases in both vertebrates and invertebrates have been coopted by the NMJ; the particular synaptic effects of neuregulin presumably result from localization of ligands and receptors, specific combinations of signaling intermediates, and the transcriptional targets competent to be activated in synaptic nuclei.

Extrasynaptic Repression

Shortly after AChRs begin to cluster at the synapse, the density of extrasynaptic AChRs begins to decline. The density increases again following denervation, resulting from increased transcription of AChR subunit genes by extrasynaptic nuclei (Merlie et al 1984, Tsay & Schmidt 1989). Two explanations for this phenomenon were debated during the 1960s and 1970s: that the nerve supplied a repressive factor to the muscle, or that electrically active muscle synthesized

less AChR than did inactive muscles. Eventually, compelling evidence for a predominant role of activity was provided by Lomo and others: Direct electrical stimulation of denervated muscle prevented or reversed denervation supersensitivity, whereas pharmacological blockade of synaptic transmission induced AChR synthesis in innervated muscle (reviewed in Lomo & Westgaard 1975, Fambrough 1979). Subsequently, electrical activity of muscle fibers was shown to have a repressive effect on AChR gene expression, thus providing a satisfying explanation of the phenomenon (Goldman et al 1988). It is also clear that electrical activity regulates AChR levels in embryonic myotubes, both in vitro and in vivo (Burden 1977, Klarsfeld & Changeux 1985, Goldman et al 1988).

In a sense, then, the nerve does control extrasynaptic AChR density by means of a chemical mediator—it is just that the mediator is the neurotransmitter, the receptor is the AChR itself, and the mechanism is that of conventional synaptic transmission. Thus, AChRs are subject to negative regulation by their ligand, with the interesting twist that the negative effects are exerted at a distance, and the synaptic receptors responsible for initiating the signal are themselves immune to the repressive effects of activity.

Activity Signaling

The initial steps in the signaling pathway for extrasynaptic repression are those by which synaptic transmission causes muscle contraction: Release of acetylcholine activates the AChR, leading to a depolarizing synaptic potential, which reaches threshold and triggers an action potential. The action potential propagates the repressive signal of depolarization along the length of the muscle fiber, leading to increased levels of cytosolic calcium, by efflux from the sarcoplasmic reticulum and influx through the plasma membrane. The calcium then activates muscle contraction and represses AChR gene transcription (Huang et al 1994a, Adams & Goldman 1998).

Following calcium mobilization, pathways of excitation-contraction and excitation-transcription coupling diverge. Whereas binding of calcium to troponin is critical for contractility, the key target for AChR gene expression is protein kinase C (PKC) (Klarsfeld et al 1989, Huang et al 1992). This serine/threonine kinase is activated by calcium and phospholipids, so it might be activated directly by calcium influx or indirectly by calcium-activated production of diacylglycerol. Critical targets of PKC, in turn, are the myogenic factors, transcriptional activators required for initiation of the entire myogenic program, including the initial induction of AChR subunit genes (reviewed in Rudnicki & Jaenisch 1995). These factors (myoD, myf5, MRF4, and myogenin) all bind to a short sequence called an E box that is present, along with the N boxes mentioned above, and other critical sites (Bessereau et al 1998) in AChR subunit genes. E box-containing sequences in several AChR subunit

genes confer activity-dependent expression on reporters (Piette et al 1990; Prody et al 1992; Chahine et al 1992; Simon & Burden 1993; Tang et al 1994; Walke et al 1996a,b). Activity-dependent, PKC-mediated phosphorylation of the myogenic factors renders them inactive in the short term and leads to their transcriptional downregulation over a longer period (Eftimie et al 1991, Huang et al 1994b). Thus, the transcriptional machinery responsible for turning on AChR genes early in development comes under the control of electrical activity at a later stage.

Other Postsynaptic Components

Are mechanisms responsible for localizing AChRs to synapses used by other synaptic molecules? In broad terms, the nerve appears to use the three mechanisms described above—agrin- and rapsyn-dependent clustering, synapse-specific transcription, and activity-dependent repression—to regulate many components of the posystnaptic apparatus. Space limitations preclude a complete discussion of this issue, but the following are some general points: 1. Agrin causes clustering of many synaptic proteins in cultured muscle cells, including components of the plasma membrane, cytoskeleton, and basal lamina (Wallace 1989, Bowe & Fallon 1995), and all aspects of postsynaptic differentiation are impaired in agrin$^{-/-}$ and MuSK$^{-/-}$ mice (DeChiara et al 1996, Gautam et al 1996). 2. Rapsyn is required for clustering of many of these proteins, but not for MuSK, laminin ß2, or acetylcholinesterase (Gautam et al 1995, Moscoso et al 1995a, Apel et al 1997). These latter proteins may therefore be components of the primary scaffold mentioned above, to which rapsyn recruits additional members. Interestingly, both laminin ß2 and acetylcholinesterase appear to bear localizing signals that target them to synaptic sites (Rotundo et al 1997, Martin et al 1995). 3. RNAs encoding several synaptic proteins are concentrated at synaptic sites, suggesting that the cognate genes are selectively transcribed by synaptic nuclei. These include MuSK, N-CAM, a protein kinase A subunit and acetylcholinesterase (Moscoso et al 1995b, Valenzuela et al 1995, Imaizumi-Scherrer et al 1996). 4. Levels of several components, including MuSK and N-CAM, are regulated by activity in parallel with AChRs (Covault & Sanes 1985, Valenzuala et al 1995). For acetylcholinesterase, however, electrical activity exerts a positive rather than a negative effect (reviewed in Massoulie et al 1993). Thus, similar signals are combined in different ways for various gene products, resulting in different patterns of expression.

PRESYNAPTIC DIFFERENTIATION (FIGURE 4)

Growth cones can release neurotransmitter in response to electrical stimulation even before they make contact with muscle fibers (Young & Poo 1983, Hume

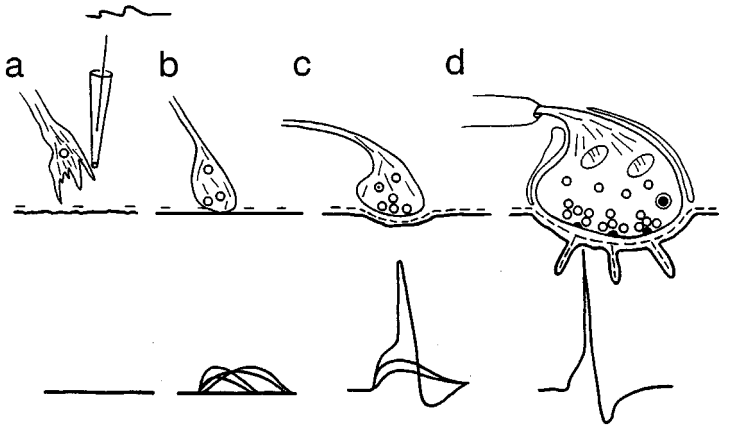

Figure 4 Differentiation of the motor nerve terminal. (*a*) Even before the motor axon contacts a myotube, its growth cone can release quanta (vesicles) of acetylcholine in response to depolarization. (*b*) Transmission at newly formed nerve-muscle contacts is weak and subthreshold. (*c*) Later in embryogenesis, vesicles accumulate in the nerve terminal and quantal content increases, but transmission remains prone to failure and the action potential is broad. (*d*) In the adult, the preterminal axon is myelinated, and transmission has a high safety factor.

et al 1983). Moreover, spontaneous and evoked neuromuscular transmission begin within minutes after nerve contacts muscle (Kidokoro & Yeh 1982, Chow & Poo 1985, Xie & Poo 1986, Evers et al 1989). Nonetheless, the synapses are initially very weak, not only because AChR density is low, but also because very little transmitter is released (Kullberg et al 1977, Nakajima et al 1980). Over the subsequent days, as the postsynaptic membrane matures, the presynaptic terminal also differentiates dramatically.

Nerve terminals at newly formed synapses are simple bulbous enlargements (Linden et al 1988), sometimes formed en passant rather than by growth cones (Sheard & Duxson 1997). They bear few synaptic vesicles and no ultrastructurally recognizable active zones. Subsequently, synaptic vesicles increase in number, and cytoskeletal elements characteristic of the axon are lost. Active zones appear, vesicles become clustered at the active zones, and the nerve terminal becomes polarized (Kelly & Zacks 1969, Takahashi et al 1987, Ko 1985, Buchanan et al 1989, Lupa & Hall 1989). These changes, accompanied by a parallel increase in synaptic volume and area, underly large increases in the frequency of spontaneous exocytosis and the number of vesicles that release neurotransmitter in response to stimulation (Dennis 1981).

The observations that presynaptic specializations form only at sites of contact with muscle fibers, in normal muscle, following reinnervation and in

nerve-muscle cocultures imply that target-derived factors organize presynaptic differentiation (Lupa et al 1990). The factors that stimulate presynaptic differentiation are unknown, but three proteins present in synaptic basal lamina exhibit activities consistent with their playing roles: Beads coated with fibroblast growth factor 2 (FGF2) stimulate local clustering of synaptic vesicles when applied to neurites, and substrata containing agrin or synaptic isoforms of laminin cause neurites to stop growing and to accumulate synaptic vesicles (Dai & Peng 1995; Porter et al 1995; Patton et al 1995; Campagna et al 1995, 1997b; Chang et al 1997). Additional candidates have emerged from studies in vitro that were conducted with other aims in mind. For example, Poo and others have shown that neurotrophins are released from muscle cells and enhance the efficacy of transmitter release from newly formed synapses (reviewed in Lu & Figurov 1997, Fitzsimonds & Poo 1998). In addition, numerous trophic agents and cell adhesion molecules expressed by muscle have been shown to promote differentiation of isolated motoneurons (reviewed in Oppenheim 1996, Dai & Peng 1996).

Unfortunately, there is no evidence at present that any of these factors are critical for the initial stages of presynaptic differentiation in vivo. Maturation of nerve terminals is greatly impaired in mutant mice that lack laminin ß2 (see below), but the initial stages of differentiation proceed normally (Noakes et al 1995). Few neuromuscular defects are detectable in mutant mice lacking N-CAM, which promotes motor neurite outgrowth, or FGF5, which is a potent inducer of choline acetyltransferase in vitro (Moscoso et al 1998). Presynaptic differentiation is impaired in agrin$^{-/-}$ mice, but this defect is apparently secondary to a blockade of postsynaptic differentiation: In the absence of agrin, myotubes do not properly synthesize or localize a still-unidentified retrograde factor (Burgess et al 1998).

Whatever the signals that induce presynaptic differentiation, it appears likely that they act at least in part by triggering an increase in intracellular calcium levels (Dai & Peng 1993). In addition, synapsins, vesicle-associated proteins that modulate transmitter release, may regulate the structural and functional maturation of motor nerve terminals (Lu et al 1996). A major question is whether maturation involves changes in gene expression or only rearrangement of preexisting synaptic components. The observations that vesicles are present before synapses form and that different branches of a single axon respond independently to targets (Wang et al 1998) argue that posttranslational steps predominate. On the other hand, expression of genes encoding vesicle proteins increases soon after synaptogenesis begins, and motoneurons synthesize different isoforms of some vesicle proteins before and after their axons contact targets (Lou & Bixby 1995, Campagna et al 1997a, Plunkett et al 1998, reviewed in Sanes & Scheller 1997). Thus, both changes in gene expression and

redistribution of preexisting components probably contribute to presynaptic as well as to postsynaptic differentiation.

SYNAPTIC MATURATION (FIGURES 5 AND 6)

The mammalian NMJ undergoes dramatic changes in structure and function during the first few postnatal weeks. Perhaps most striking are changes in the shape of the synapse. Initially, all of the axons that innervate each muscle fiber are intertwined atop an elliptical, uniform plaque of AChRs. Then, as inputs are eliminated (see below), spots of low AChR density appear as perforations within the plaque. Sculpting continues until the postsynaptic apparatus acquires a pretzel-like form (Nystrom 1968, Steinbach 1981, Slater 1982, Balice-Gordon et al 1993). Interestingly, the spatial correspondence between nerve terminals and the AChR-rich membrane is considerably more precise in the pretzels than in the plaques, suggesting that pre- and postsynaptic specializations become increasingly interdependent as development proceeds.

The branched terminal arbor forms during the second postnatal weeks in rodents, when the animal is only a fraction of its adult size. Subsequent growth of muscles occurs by increases in muscle fiber length and diameter but not fiber number. The NMJ grows in parallel with the muscle fiber during this period, but synaptic geometry changes little: Preexisting branches undergo intercalary growth throughout their length, and few branches are added or lost. Thus, whereas early stages of synaptogenesis require active signaling between nerve and muscle, synaptic growth may result in large part from the tight adhesion of axon terminals to passively expanding postsynaptic sites (Balice-Gordon & Lichtman 1990, Balice-Gordon et al 1990).

As synaptic topography matures in the x and y dimensions, the postsynaptic apparatus becomes more complex in the z dimension (Desaki & Uehara 1987). First, the membrane becomes depressed into shallow gutters beneath the nerve terminals. Subsequently, gutters invaginate to form junctional folds, which invariably lie directly across from the active zones in the nerve terminal. The mechanisms that underly fold formation are unknown, but the adhesive forces that appear to underly intercalary growth can also account for the formation of gutters and folds, and for the apposition of folds to active zones (MJ Marques & JW Lichtman, in preparation; Figure 6*a*).

As folds form, the entire postsynaptic apparatus becomes raised above the smooth cylindrical surface of the muscle fiber, an appearance that gave rise to the term “endplate.” Within the specialized cytoplasm of the endplate are the synaptic nuclei, described above, and a biochemically specialized Golgi apparatus that may be dedicated to the processing of synaptic components (Antony et al 1995, Jasmin et al 1995). Thus, structural maturation of the endplate may

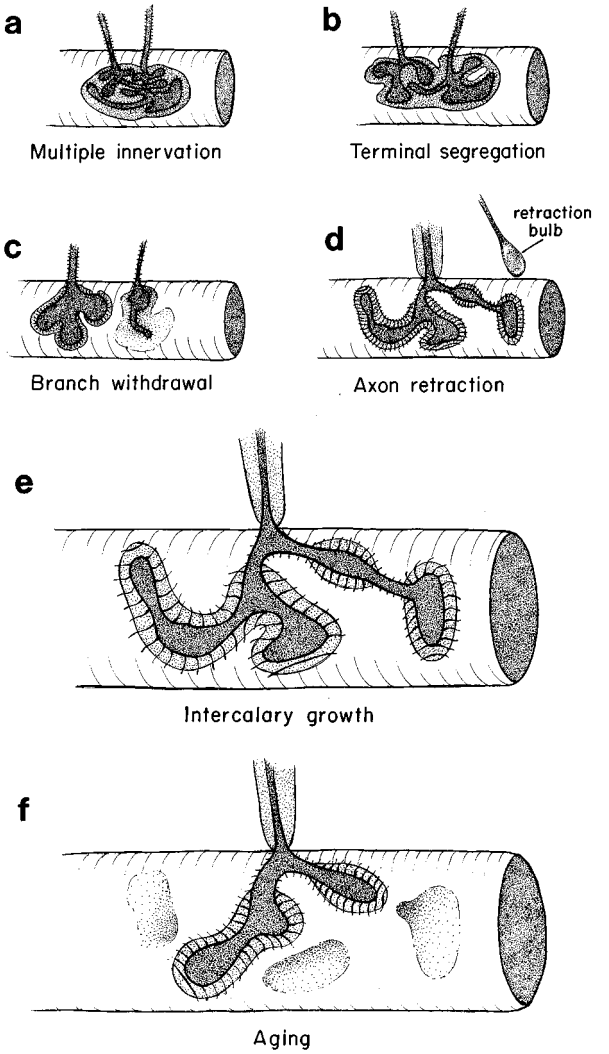

Figure 5 Postnatal maturation of the neuromuscular junction. (*a*) The muscle fiber is multiply innervated at birth, with all inputs sharing a plaque-shaped postsynaptic domain. (*b*) As synapse elimination proceeds, terminals from competing inputs become segregated and the plaque acquires perforations. (*c*) By two weeks after birth, in rodents, all inputs save one have retracted from each muscle fiber and the postsynaptic apparatus has formed a convoluted arbor whose outlines correspond precisely to those of the remaining nerve terminal. (*d*) Subsequently, coordinate expansion of the nerve terminal and muscle fiber lead to growth of the NMJ without major change in synaptic geometry. (*e*) As animals age, some portions of the NMJ are lost, leading to a varicose appearance.

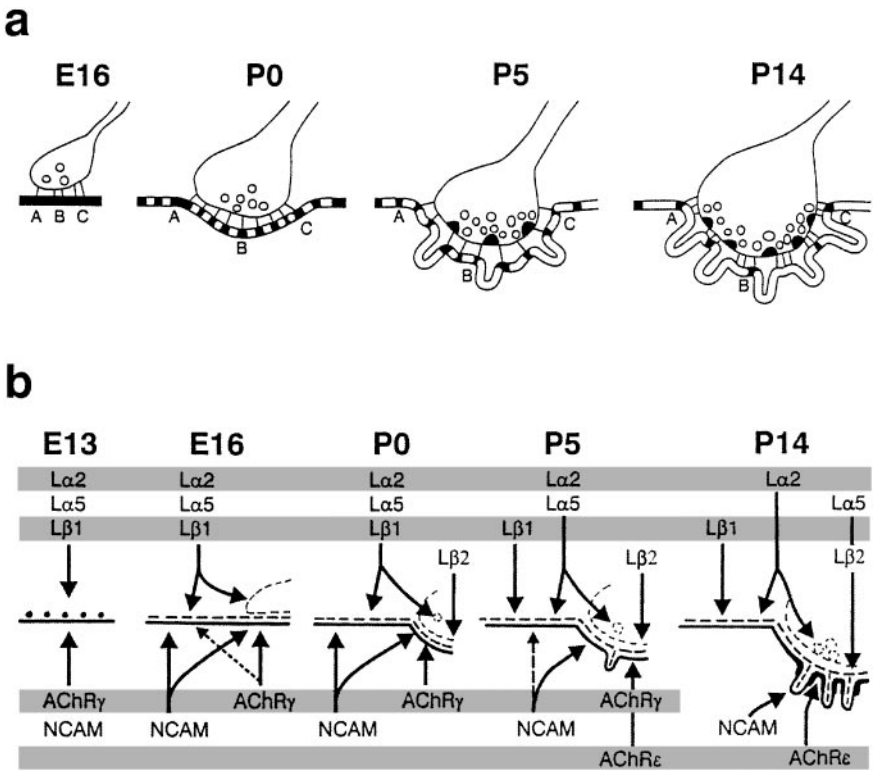

Figure 6 Maturation of the postsynaptic apparatus. Ages, which are approximate, refer to embryonic (*E*) and postnatal (*P*) days in the mouse. (*a*) Formation of synaptic gutters is followed by formation of junction folds. The sketches show a plausible mechanism for gutter and fold formation in which both result from passive growth of the muscle fiber, constrained by the presence of bonds that link the pre- and postsynaptic membranes via the basal lamina. The original membrane is shown in black, and capital letters mark corresponding points at several ages. (*b*) The molecular composition of the postsynaptic apparatus changes as it matures. As examples, these sketches show the laminin (*L*) alpha and beta subunits of the basal lamina, the AChR gamma and epsilon subunits of the postsynaptic membrane, and N-CAM (Covault & Sanes 1986, Missias et al 1996, Patton et al 1997).

underly an increasingly efficient compartmentalization of synthetic capabilities within the muscle fiber.

Changes in the shape of the postsynaptic apparatus are accompanied by numerous alterations in its molecular architecture, a small subset of which are illustrated in Figure 6*b* (see also Bewick et al 1996, PT Martin et al 1996, Patton et al 1997). Best studied is the conversion of AChRs from an embryonic to an adult form: Synaptic AChRs containing a gamma subunit ($\alpha_2\beta\gamma\delta$) are replaced

by AChRs containing a homologous epsilon subunit ($\alpha_2\beta\varepsilon\delta$) during the first postnatal week (Mishina et al 1986, Gu & Hall 1988, Missias et al 1996). The basis of the substitution is transcriptional: Synaptic nuclei downregulate γ-gene expression and activate the ε-gene shortly after birth (Martinou & Merlie 1991, Sanes et al 1991, Missias et al 1996). The switch appears to be initiated by the nerve, but no ε-specific activator has yet been described. One possibility is that the switch is part of the general maturational program of muscle fibers, much like the switch in expression from fetal to adult myosins. The functional significance of the switch may be in the different kinetics of ε- and γ-containing AChRs (Schuetze & Role 1987, Villarroel & Sakmann 1996). The transition may also be important for structural maturation of the synapse: in epsilon$^{-/-}$ mutant mice, γ-containing AChRs are retained, and formation of the raised endplate and of junctional folds is impaired (Missias et al 1997).

In addition to changing in subunit composition, AChRs become stabilized perinatally: They become resistant to dispersal by calcium chelators and collagenase (Bloch et al 1986), and their half-life in the postsynaptic membrane increases by an order of magnitude, from approximately 1 day to $>$10 days (reviewed in Salpeter & Loring 1985). The molecular basis of the stabilization remains unclear, and it seems unlikely that there is any simple correspondence between stability and subunit composition (Shyng et al 1991, Caroni et al 1993, O'Malley et al 1997). An attractive possibility is that stabilization reflects a change in the association of AChRs with basal lamina or cytoskeletal components. For example, agrin binds to dystroglycan (see above), which in turn associates with a cytoskeletal complex that includes dystrophin, utrophin, and dystrobrevin (Figure 1). This complex matures postnatally (Bewick et al 1996, Grady et al 1997a) and appears to be inessential for initial AChR clustering (Bowen et al 1996, Gesemann et al 1996, Grady et al 1997b, Deconinck et al 1997) but may be involved in the growth or stabilization of clusters (Campanelli et al 1994, Namba & Scheller 1996, Xu & Salpeter 1997, Zhou et al 1998). In any event, AChRs stabilize before the period of rapid synaptic growth and synapse elimination, so metabolic stabilization is not likely to underly morphological stabilization.

Schwann cells also mature postnatally. At birth, terminal Schwann cells form a loose cap over groups of axonal boutons at each synaptic site, whereas each terminal bouton is separately capped in adults (Hirata et al 1997). Perhaps more importantly, preterminal Schwann cells are present in embryos, but do not form myelin until postnatally. In addition, both preterminal and terminal Schwann cells are acutely dependent on axonal contact for their survival in neonates, but become relatively nerve-independent postnatally (Trachtenberg & Thompson 1996).

Postnatal changes in the structure of the NMJ have profound effects on its function. Accumulation of synaptic vesicles and active zones in the nerve

terminal leads to an increase in quantal content and a decrease in the susceptibility of the terminal to fatigue. Formation of myelin speeds conduction of the action potential along axons. The formation of folds, and the accumulation of sodium channels at synaptic sites enhance the speed and reliability of the postsynaptic response to released transmitter. Finally, maturation of the basal lamina binds the pre- and postsynaptic cells together strongly enough to withstand up to a century of constant use.

SYNAPSE ELIMINATION (FIGURES 5 AND 7)

Most vertebrate muscle fibers are innervated by more than one motor axon during a protracted developmental period. All inputs but one are withdrawn during early postnatal life in a process called synapse elimination. This term is unfortunate because the size of the endplate and the numbers of AChRs, junctional folds, and active zones all increase during this period (see above). "Input elimination" therefore describes the phenomenon more accurately, but the older term is too entrenched to be changed.

Although synapse elimination is often diagrammed as a stereotyped and simple process, it is actually variable in several respects. First, only fibers that generate action potentials in response to nerve stimulation (twitch fibers) undergo synapse elimination. Tonic muscle fibers, which contract slowly and do not generate action potentials, have multiple synaptic sites distributed along their length and retain multiple axons at individual sites (Ridge 1971, Gordon & Van Essen 1983, Lichtman et al 1985, Porter & Baker 1996). Thus, synapse elimination is restricted to muscle fibers that ultimately receive strong synaptic input from a single motor axon. Second, even for twitch fibers, the efficiency of the process that removes multiple innervation differs between species: $>1/3$ of endplates maintain more than one axon in some amphibian muscles. Finally, the maximum number of inputs varies, among muscles (reviewed in Jansen & Fladby 1990). Despite this variability, however, common mechanisms appear to underly synapse elimination in all muscles.

Polyneuronal innervation originates by the convergence of multiple motor axons on individual muscle fibers. All inputs to each fiber form their synapses at the single site pioneered by the first input. This pattern, along with the initial susceptibility of the entire myotube to innervation, suggests that extrasynaptic portions of the muscle rapidly become refractory to innervation following the onset of synaptogenesis (Bennett & Pettigrew 1976); direct evidence for innervation-dependent refractoriness to hyperinnervation has come from studies of reinnervation (Frank et al 1976). Loss of multiple innervation is due to retraction of some terminal branches from each muscle fiber without any change in the number of motor axons innervating the muscle as a whole (Brown et al 1976, Balice-Gordon & Thompson 1988). Thus, the size but not the number of motor

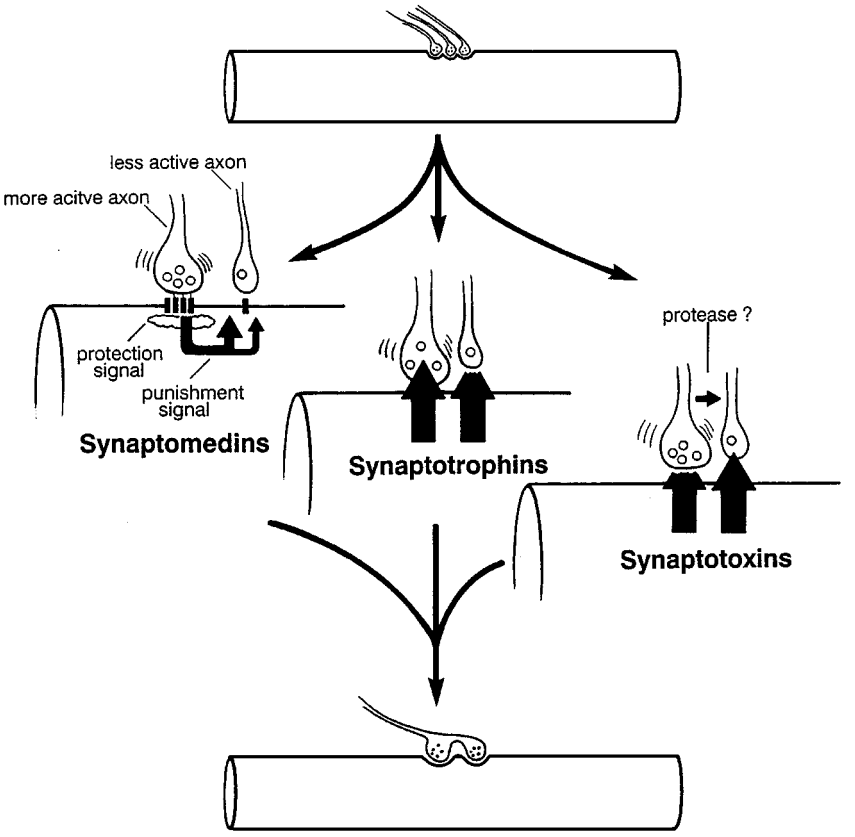

Figure 7 Signals that might drive synapse elimination. The muscle fiber mediates elimination, and the relative activity of inputs to a muscle fiber affects their fate. *Synaptomedins* could render regions within the postsynaptic apparatus differentially adhesive to competing axons. *Synaptotrophins* might be needed for maintenance of nerve terminals and be more effectively internalized or utilized by active terminals. *Synaptotoxins* might promote withdrawal, and active terminals might be relatively resistant to their negative effects. See text for explanation and references.

units decreases during synapse elimination. Some motoneurons do die during normal development, but the period of cell death precedes the period of synapse elimination. Transient polyneuronal reinnervation and synapse elimination also occur following reinnervation of denervated adult muscle fibers (Ostberg et al 1986, Rich & Lichtman 1989, Werle & Herrera 1991, Barry & Ribchester 1995).

The process of synapse elimination is gradual in two ways. First, within a single muscle, the time at which individual fibers lose inputs is spread out over many days. In the rat diaphragm, for example, about 5–15% of the fibers

become singly innervated on each postnatal day until all are singly innervated at two weeks of age (Redfern 1970, Rosenthal & Taraskevich 1977). Second, the transition from multiple to single innervation is gradual at single NMJs: The strength of each input is roughly equal at birth, but one input gradually gains territory as the others lose (Balice-Gordon & Lichtman 1993, Colman et al 1997). Anatomically, the process occurs as a stepwise loss of branches. Each branch undergoes a program of atrophy, detachment, and withdrawal, leading to progressive separation of the territories that each axon occupies. Finally, one axon forms a "retraction bulb," and withdraws from the end-plate (Riley 1977, 1981; Bixby 1981; Balice-Gordon et al 1993; W-B Gan & JW Lichtman, 1998). The loss of territory by an axon is contemporaneous with and may be related to the transformation from an oval, AChR-rich plaque to a pretzel-shaped structure (Balice-Gordon & Lichtman 1993; Figure 5*a–c*). Physiological signs of axonal competition are subtle (Rosenthal & Taraskevitch 1977), but quantal responses clearly decrease in both size and number prior to complete axon withdrawal (Dunia & Herrera 1993, Colman et al 1997). Junctions with two equally efficacious inputs can persist for many days in mice, but once the balance tips in favor of one input, the process of skewing accelerates (Colman et al 1997). Likewise, in frogs, multiple innervation sometimes persists into adulthood in fibers with evenly matched inputs (Werle & Herrera 1987, 1988; Herrera & Werle 1990).

Synapse elimination is competitive in the sense that the fate of one axon's terminals depends on the presence or absence of its neighbors (Colman & Lichtman 1993). The central question in synapse elimination is the nature of the signals that mediate the competition. Any mechanism must account for two fundamental characteristics of the competitive process. The first is that the muscle mediates competitive interactions among axons. The most compelling evidence for this idea is that axons compete even when they are separated from each other by long distances. For example, following reinnervation of a muscle by two nerves, individual muscle fibers become innervated at multiple sites, but then eliminate one endplate if it is up to 1 mm from another (Kuffler et al 1997). Second, electrical activity affects the intensity of competition. For example, blockade of synaptic activity and excessive stimulation slow and speed elimination, respectively, implying that the rate at which muscles become singly innervated is positively correlated with their overall level of activity (Duxson & Vrbova 1985, Thompson 1985, Barry & Ribchester 1995). These results are consistent with the view that differential activity determines the winner and the loser of the competition, but may also reflect the requirement of activity for maturation of muscle generally and the NMJ in particular.

At least three general mechanisms could explain the competitive nature, spatial constraints, and activity-dependence of synapse elimination (Figure 7).

First, muscles might release limiting amounts of a maintenance factor (a synaptotrophin; Snider & Lichtman 1996) for which axons compete. The more active axon would be better able to take up or utilize the factor, whereupon it would become even stronger and more active, until eventually it starved its competitor. In a more sophisticated version of the model, both release from muscle and uptake by nerve would be activity-dependent, so that the axon better able to bring the muscle fiber to threshold would be selectively rewarded. In fact, several factors produced by muscles are capable of retarding synapse elimination when applied to postnatal muscles; these include leukemia inhibitory factor, BDNF, CNTF, FGF-2, androgenic steroids, IGF-1, and GDNF (Caroni & Becker 1992; Kwon et al 1995; Kwon & Gurney 1996; Jordan et al 1995; Jordan 1996; English & Schwartz 1995; Nguyen et al 1998). Unfortunately, interpretation of these experiments is made complicated by relatively nonspecific effects of such treatments. For example, CNTF exerts a cachectic effect on skeletal muscle that may delay maturation (D Martin et al 1996), and several of the factors cause axonal sprouting (Gurney et al 1992; Kwon & Gurney 1994; Caroni et al 1994). Thus, the idea that there is a retrogradely diffusing synaptotrophic molecule at the NMJ is attractive but unproven.

Alternatively, axons might vie to escape the punitive effects of a damaging muscle-derived synaptotoxin. Here, activity might serve to make an axon immune. One popular idea is that the toxic factor is a protease, whose activity is held in check by endogenous inhibitors. Specifically, Vrbova and colleagues have proposed that calcium-activated neutral proteases destabilize nerve terminals (Connold et al 1986, Tyc & Vrbova 1995, Swanson & Vbova 1987), whereas Festoff, Nelson, and colleagues have argued that thrombin, a serine protease, plays a key role (Liu et al 1994; Zoubine et al 1996; Glazner et al 1997). Moreover, proteases could mediate direct competitive interactions between axons, as well as retrograde effects of muscle on nerve. Interestingly, agrin may function as a protease inhibitor (Biroc et al 1993).

A third model also involves retrograde influences, but postulates that their precise localization within a synaptic plaque allows them to differentially reward or punish the competing axons. This, in turn, requires that there be intracellular signals, synaptomedins, that act within the muscle fiber to localize the sites at which the intercellular signals are presented. Key to the synaptomedin model is that some inhomogeneity within the postsynaptic apparatus anticipates the loss of a particular nerve terminal. In fact, levels of AChRs, rapsyn, and utrophin begin to decline at sites of synapse removal before the overlying nerve withdraws (Rich & Lichtman 1989a; Balice-Gordon & Lichtman 1993; Hesselmans et al 1993; Culican et al 1998). These localized alterations occur within a postsynaptic plaque, and make plausible the idea that inhomogeneities are involved in synapse elimination.

Evidence for the synaptomedin model came from studies designed to assess roles of differential activity in synapse elimination. Initial experiments involved paralyzing or stimulating a subset of axons to a muscle, then asking whether the active or inactive axons were favored (Ridge & Betz 1984, Callaway et al 1989, Ribchester 1988). Unfortunately, these studies reached inconsistent conclusions. In addition, the results were difficult to interpret, because alteration of activity in a subset of axons ended up affecting the overall activity of many muscle fibers, thereby confounding permissive and instructive effects of activity. To circumvent this problem, Balice-Gordon & Lichtman (1994) focally applied the irreversible receptor antagonist α-bungarotoxin to a small part of a singly innervated NMJ, thereby inactivating a subset of postsynaptic sites within a single endplate. Blockade of small regions within a NMJ resulted in localized loss of both the blocked AChRs and the directly overlying terminal boutons. When larger regions ($>60\%$) of the junction were blocked, however, neither pre- nor postsynaptic changes were seen. These results imply that active synaptic sites cause the elimination of neighboring inactive sites. One possible explanation is that active sites generate two distinct local signals or synaptomedins: one that spreads over short distances to disassemble inactive sites, and a more highly localized one that protects the active site itself (Nguyen & Lichtman 1996).

Consistent with this idea is the otherwise puzzling observation that AChRs are lost from denervated portions of incompletely reoccupied NMJs following reinnervation (Aframian & Grinnell 1988, Werle & Sojka 1996, Stanco & Werle 1997, Astrow et al 1996), whereas AChRs at completely denervated junctions are relatively stable (Frank et al 1976, Moss & Schuetze 1987). Apparently, inactive parts of a postsynaptic apparatus are stable in isolation but disappear when they are adjacent to active regions. This sort of activity dependence is different from so-called Hebbian competition, in which active synapses are rewarded directly; in synapse elimination, active synapses prosper by punishing their inactive neighbors (Lichtman & Balice-Gordon 1990).

What might the synaptomedins be? The punishment signal is likely to be initiated by depolarization, acting via a second messenger, such as calcium or a phosphatase (Dai & Peng 1998). Evidence that action potentials are key comes from an observation mentioned above: Only fibers that generate action potentials undergo synapse elimination. On the other hand, action potentials may not be sufficient to trigger synapse loss because, as also mentioned above, ectopic synapses spaced >1 mm apart can be permanently maintained on a muscle fiber even though action potentials propagate along the entire fiber. Completely obscure for the moment are the identity of the mechanism that effects the punishment, the targets of punishment, and the nature of the protection pathway.

Finally, the synaptomedin hypothesis requires that a retrograde signal pass from a small portion of the endplate to an overlying nerve terminal. Such signals

could be synaptotrophins or synaptotoxins, with their release or range sharply restricted. Another possibility is that the signal is adhesion per se. In that pre- and postsynaptic membranes adhere tightly to each other, breakage of adhesive bonds is prerequisite to retraction of the losing axon. It is attractive to imagine that local loss or down-regulation of an adhesive molecule could induce, as well as permit, loss of nerve terminals.

SYNAPSE MAINTENANCE (FIGURE 8)

Mature NMJs persist for the life of the animal. Is this stability real or apparent? One view is that neuromuscular synapses constantly turn over. This idea arose in part from the observation that adult nerve terminals sprout beyond the confines of the endplate when rendered inactive (reviewed in Brown et al 1981), possibly because components of the developmental growth-promoting program can readily be reactivated (Caroni 1997). Thus, synapses might be in a state of dynamic equilibrium, with branches frequently sprouting and retracting.

In fact, mature mammalian endplates do contain small numbers of vacant postsynaptic gutters, terminal sprouts, and degenerating axonal profiles (Barker & Ip 1966, Cardasis & Padykula 1981, Kawabuchi et al 1995). Likewise, branches of frog nerve terminals occasionally extend beyond or fail to completely fill postsynaptic gutters (reviewed in Wernig & Herrera 1986, Herrera & Werle 1990). Time-lapse imaging in frogs shows that most NMJs undergo remodeling at some of their branches, while other branches within the same endplate are stably maintained (Langenfeld-Oster et al 1993, Ko & Chen 1996). Changes in the extracellular matrix and Schwann cells may precede synaptic remodeling (Chen et al 1991, Chen & Ko 1994). It remains unclear whether sprouting and retraction are mechanistically linked, so that one calls forth the other, or whether they are regulated independently, with levels of both so low and similar that the size of endplates does not change much in normal adult muscle.

In contrast to the situation in frogs, time lapse imaging in mice shows that most endplates are remarkably stable: NMJs neither add nor lose branches over significant portions of an animal's life even though junctions enlarge considerably as the animal grows (Lichtman et al 1987, Balice-Gordon & Lichtman 1990, Balice-Gordon et al 1990, Wigston 1990). Thus, synaptic turnover is not a necessary feature of synaptic maintenance. The stability of adult mammalian endplates could reflect either an inert self-sufficiency or require ongoing maintenance. Experiments on denervated and damaged muscles support the former alternative. Following axotomy, the nerve terminal degenerates and the Schwann cell retracts from the synaptic site, yet elaborate postsynaptic specializations persist for long periods (Frank et al 1976). Thus, the postsynaptic apparatus can

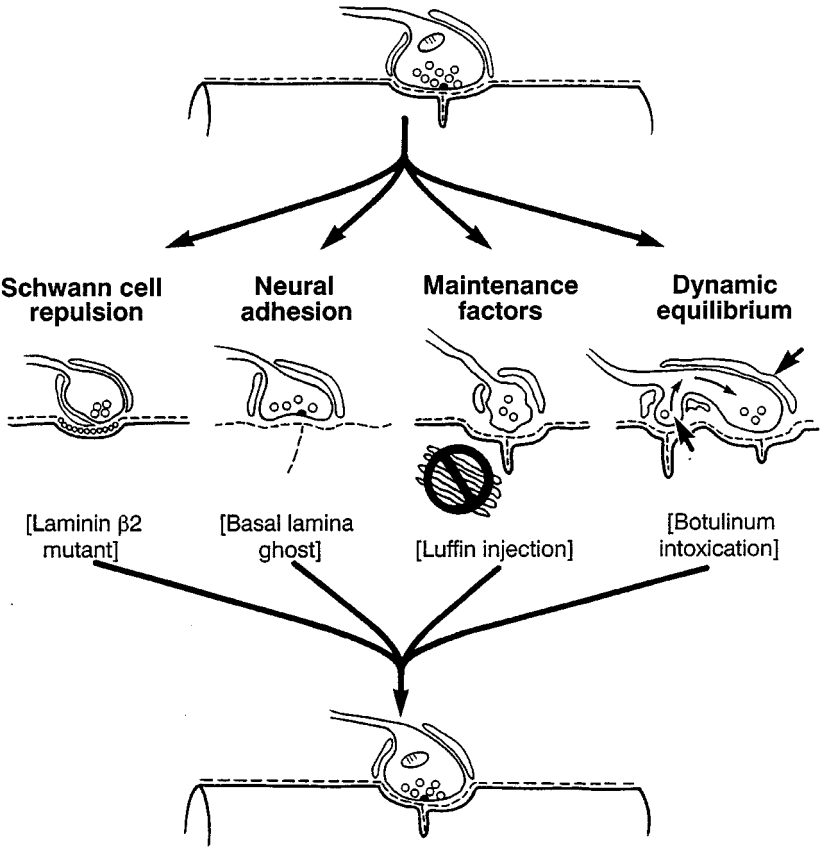

Figure 8 Mechanisms that might account for the maintenance of synaptic structure in adults, with experimental results that support each of four alternatives. Basal lamina actively repels Schwann cell processes from the synaptic cleft, and mediates adhesion of nerve terminals. Thus, Schwann cells invade the cleft in laminin $\beta 2$ mutants, and nerve terminals adhere to synaptic basal lamina in the absence of the muscle fiber. Muscles also provide a constant source of maintenance factors, as shown by the partial retraction of terminals following injection of a membrane-impermeable protein synthesis inhibitor, luffin, into muscle fibers. Finally, in some cases, stability may be illusory: Nerve terminals can retract from some sites and sprout to form new sites; if these processes are in equilibrium, the average size and shape of synapses remains constant.

be maintained in the absence of other synaptic cells. Components of synaptic basal lamina induce these specializations when myotubes regenerate following injury (Burden et al 1979, Goldman et al 1991, Jo & Burden 1992, Brenner et al 1992), so it seems reasonable to assume that synaptic basal lamina components are at least partially responsible for the maintanentance of the postsynaptic apparatus even when the nerve is present. Conversely, nerve terminals persist at synaptic sites on basal lamina sheaths in frogs following degeneration of muscle fibers (Sanes et al 1978, Tal & Rotshenker 1983, Dunaevsky & Connor 1998). These results demonstrate that maintenance of the postsynaptic apparatus does not require ongoing intracellular signaling and supports the idea that synaptic maintenance is passive.

In addition to maintaining the nerve terminal and muscle fiber, synaptic basal lamina sends an inhibitory signal to the terminal Schwann cell. In normal muscle, Schwann cells cap nerve terminals, but in mutant mice lacking the synaptic laminin $\beta 2$ chain, Schwann cell processes invade the synaptic cleft and impair synaptic transmission (Noakes et al 1995). Although this invasion might have reflected decreased adhesion of nerve to basal lamina, subsequent studies showed that synaptic laminins activity inhibit extension of Schwann cell processes (Patton et al 1998). Thus, maintenance of normal nerve-muscle apposition requires not only tight adhesion of nerve to muscle via the intervening cleft, but active repulsion of the Schwann cell from the cleft. This interaction, in turn, may be necessary because Schwann cells need to be in close apposition to nerve terminals to provide them with trophic sustenance (Pfrieger & Barres 1997, Trachtenberg & Thompson 1997, Arce et al 1998). Repulsion from the cleft allows the nerve terminal–Schwann cell relationship to be intimate without becoming dysfunctional.

Although the basal lamina clearly plays important roles in synaptic maintenance, it is insufficient for complete maintenance of mammalian nerve terminals: Degeneration of mouse muscle fibers is followed within two days by withdrawal of some terminal branches from endplates (Rich & Lichtman 1989b). This result suggests either that degenerating mammalian muscle fibers release nerve- or basal lamina–destabilizing agents, or that nerve terminals require a constant supply of factors released from living fibers. An attempt to decide this question was made by injecting toxins into living muscle fibers and following the consequences over time (QT Nguyen & JW Lichtman, in preparation). Following injection of sublethal doses of a membrane-impermeant protein synthesis inhibitor, nerve terminals withdrew from many muscle fibers. This result indicates that nerve terminals may be dependent on an ongoing production of proteins by muscle fibers and raises that possibility that naturally occurring synapse elimination also involves the down-regulation of muscle-derived proteins. Such proteins might be synaptotrophins of the sort discussed above,

which could promote survival of synapses much as conventional trophic factors promote neuronal survival.

Whatever the nature of the factors that maintain synapses, their efficacy apparently declines with age. The number of AChRs per junction gradually decreases in old muscles, extrajunctional receptors appear, vacated synaptic sites and terminal sprouts become more prevelant, and some Schwann cells degenerate (Courtney & Steinbach 1981, Rosenheimer & Smith 1985, Ludatscher et al 1985, Balice-Gordon & Lichtman 1990, Boaro et al 1998). As junctions age, contiguous branches become variscose, and some retract (Figure 5*e*). These changes involve both pre- and postsynaptic disassembly and thus resemble synapse elimination rather than denervation. One possibility is that loss results from activity-mediated interactions between healthy and ineffective branches. Consistent with this idea are the observations that exercise can retard the sprouting and degeneration in old muscles, whereas inactivity can stimulate these processes (Smith & Rosenheimer 1982, Fahim 1989, Pachter & Spielholz 1990).

REGENERATION OF SYNAPSES (FIGURE 9)

Damaged adult motor axons regenerate readily and form new NMJs that look and perform much like those that form during normal development. Reinnervation of adult muscle therefore provides an accessible setting in which to analyze synaptogenesis. Indeed, many such studies, cited above, have contributed to our understanding of synapse formation and elimination. On the other hand, development and regeneration differ in several ways. Perhaps the most important is that developing axons synapse in "virgin territory," whereas regenerating axons encounter a well-differentiated terrain. This cardinal difference has several implications, on which we focus here.

First, Schwann cells accompany growing axons during embryogenesis, whereas axons regenate along pathways of Schwann cells (bands of Bungner) in perineurial tubes following nerve damage in adults (Ide 1996). At a molecular level, the interactions between the two cell types may be similar in both situations: Schwann cells express many trophic, adhesive, and growth-associated proteins as they develop, which are down-regulated in adulthood but reactivated following axotomy (Taniuchi et al 1988, Plantinga et al 1993, You et al 1997, Matsuoka et al 1997). In adults, however, these interactions serve to guide the axons to their destination, whereas in development, they play trophic but not directive roles.

A similar case can be made for terminal Schwann cells, which sprout dramatically following denervation (Reynolds & Woolf 1992). Regenerating axons grow along these processes as they return to synaptic sites, and intact axons

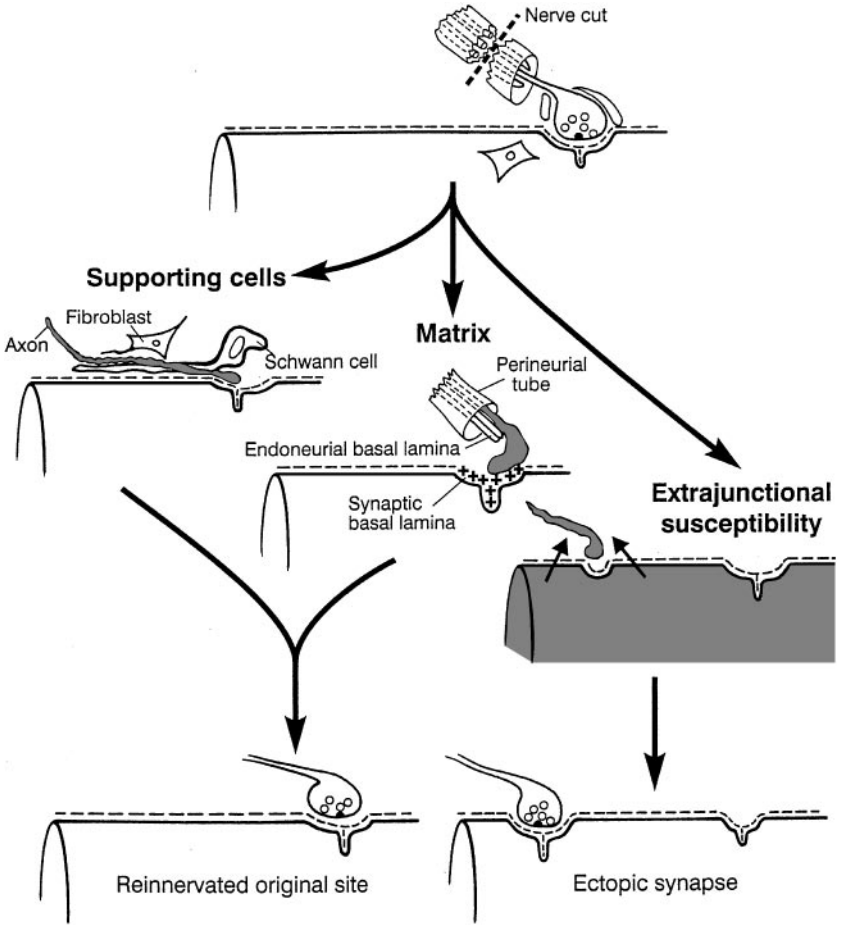

Figure 9 Motor axons selectively reinnervate original synaptic sites on denervated muscle fibers but can form "ectopic" synapses in initially nonsynaptic areas under some circumstances. Non-muscle cells may favor reinnervation of original sites: Perineurial tubes provide guidance to regenerating axons, terminal Schwann cells sprout and guide sprouting axons, and perisynaptic fibroblasts proliferate and synthesize growth-promoting matrix molecules. In addition, axons recognize components of synaptic basal lamina, as shown by the selective reinnervation of original synaptic sites on basal lamina ghosts. Extrasynaptic portions of the muscle fiber surface are normally refractory to innervation, but refractoriness is activity-dependent, and lost following denervation or paralysis. Thus, when axons are denied access to original sites, they form ectopic synapses on denervated muscle fibers.

in partially denervated muscles sprout along Schwann cell processes to reach denervated synaptic sites. Thus, Schwann cells serve as both a growth stimulus and a guidance cue for adult axons (Son & Thompson, 1995a,b; Son et al 1996; Trachtenburg & Thompson 1996, 1997). This situation differs greatly from that in developing muscle, in which axons lead Schwann cells to myotubes.

Third, denervation induces selective proliferation of interstitial cells in perisynaptic areas, which leads to localized accumulation of the adhesive matrix molecules such as tenascin-C and fibronectin (Sanes et al 1986, Connor & McMahan 1987, Gatchalian et al 1989, Caroni & Schneider 1994). These molecules may promote or direct growth of regenerating axons as they approach their targets. Interstitial cells are less strikingly concentrated near synaptic sites during development, and there are no junctional accumulations of fibronectin or tenascin-C (Sanes et al 1986, Connor 1997).

Fourth, whereas axons innervate newly formed myotubes in embryos, regenerating axons encounter fully differentiated muscle fibers. In most cases, the axons form their new synapses on a highly specialized postsynaptic apparatus at original synaptic sites (Cajal & Ramon 1928, Bennett & Pettigrew 1976, Sanes et al 1978, Rich & Lichtman 1989a). Thus, the temporal coordination of pre- and postsynaptic differentiation that characterizes development does not occur in regeneration. Perhaps because the regenerating nerve is able to reoccupy already differentiated postsynaptic sites, synapses can mature more quickly in adults than during development.

The precise reinnervation of synaptic sites is itself the final critical difference between development and reinnervation. As noted above, there is little evidence for prespecified synaptic domains on embryonic muscle fibers. Experimental analysis of reinnervation led to the demonstration that synaptic basal lamina contains cues that guide selective reinnervation of synaptic sites and differentiation of growth cones into motor nerve terminals at those sites (Sanes et al 1978, Glicksman & Sanes 1983, Kuffler 1986). These studies, in turn, motivated isolation of components such as the synaptic laminins (Hunter et al 1989b, Sanes 1995, Patton et al 1997). In view of the finding that little basal lamina is present at newly formed embryonic synapses (Kullberg et al 1977, Chiu & Sanes 1984), it was initially unclear whether these components were involved in development per se. Recently, however, genetic studies have shown that synaptic laminins are involved in the formation and maturation of the NMJ (Noakes et al 1995, Patton et al 1998). Nonetheless, as for axon–Schwann cell interactions mentioned above, the significance of nerve terminal–basal lamina actions may differ in two situations: During development, nerves localize synaptic laminins and are subsequently influenced by them, whereas during reinnervation, basal lamina components determine the site at which the synapse will form. Similarly, during development, the nerve evokes activity in muscle

that appears to render extrasynaptic membrane refractory to hyperinnervation (Bennett & Pettigrew 1976), whereas during regeneration, extrasynaptic membrane is already at least somewhat refractory to innervation (Frank et al 1975), which may help constrain synapses to original sites.

SPECIFICITY OF SYNAPSE FORMATION (FIGURE 10)

In numerous studies of neuromuscular development and regeneration, nerves have been rerouted to "foreign" muscles and muscles have been transplanted to novel sites. In virtually all cases, the motor axons successfully innervated "inappropriate" muscle fibers, leading to the conclusions that any motor axon can form NMJs on any muscle fiber and that a common set of mechanisms govern formation of all NMJs. A key feature of development, however, is that nerve-muscle connections form in stereotyped and predictable patterns: Motor neurons in coherent motor pools innervate specific muscles, "fast" motor axons innervate only "fast" muscle fibers, and so on. How, given the promiscuity of motor axons, is this specificity achieved?

If selective synapse formation is defined as requiring axonal recognition of molecular differences among muscle fibers, then two mechanisms important to the generation of specificity do not involve selectivity. In one, axons are guided to appropriate targets by cues arrayed along their paths, regardless of the molecular identity of the target itself (reviewed in Tessier-Lavigne & Goodman 1996). An apt analogy is to the motorist who proceeds along a highway and chooses an appropriate exit, confident that the signs accurately predict what will be found at the end of the ramp. Studies by Landmesser and others have shown that cues in the plexus guide motor axons to appropriate portions of the limb, sort them into muscle-specific cohorts even before they reach their targets, and even cause them to innervate inappropriate targets following surgical manipulation (Lance-Jones & Landmesser 1981, reviewed in Eisen 1994).

Another nonselective mechanism can give the appearance of choices among muscle fibers within a muscle: random innervation of initially equivalent muscle fibers followed by transsynaptic induction of specific properties. This mechanism plays a critical role in the phenomenon of fiber-type matching, whereby all muscle fibers within a motor unit are of a single type. In classical experiments, Buller and colleagues denervated neonatal muscles destined to become predominantly fast-twitch or slow-twitch, and cross-reinnervated them with each other's nerves. Remarkably, the muscles acquired contractile characteristic appropriate for their new innervation. Subsequent studies have shown that this conversion is mediated by electrical activity, but in a way that differs from that described above for AChRs: Expression levels are roughly inversely proportional to activity levels for AChR genes, whereas differing patterns of

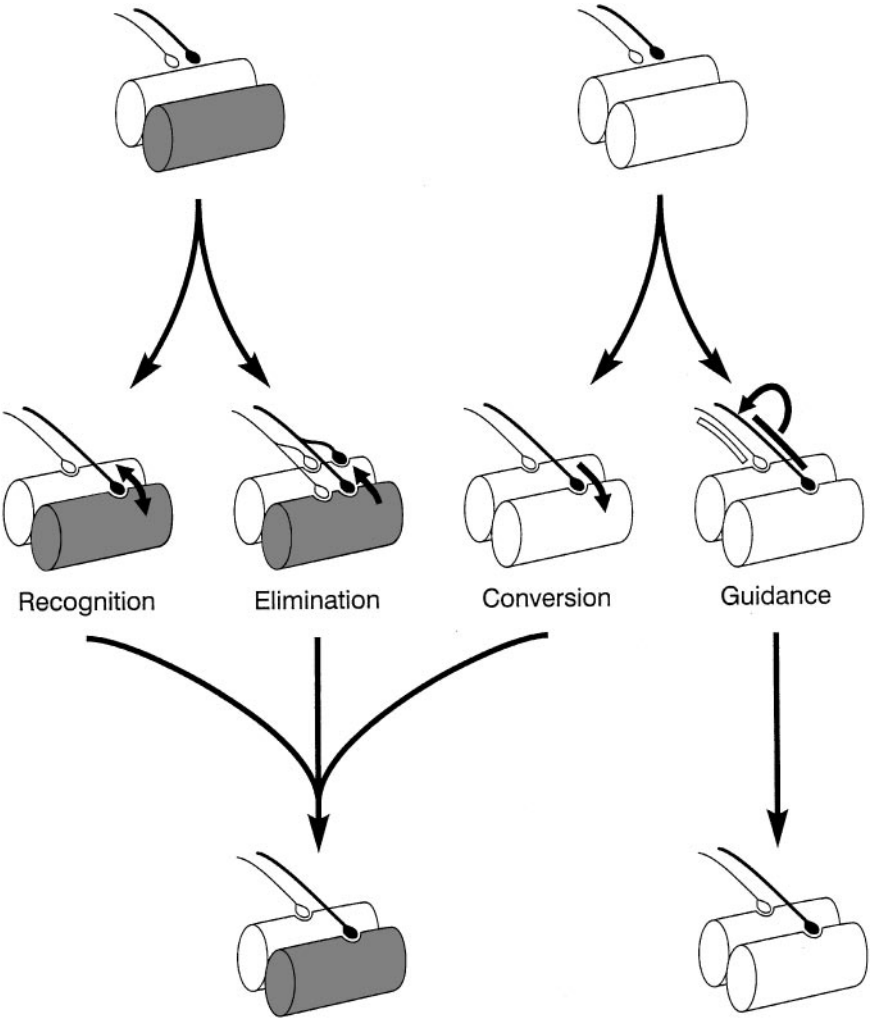

Figure 10 Four mechanisms that might account for specific matching of motor axons and muscle fibers. First, motor axons and muscle fibers might acquire their identities independently, with synaptogenesis biased in favor of molecularly matched partners. Alternatively, synapse formation might be nonselective, but matched partners might be resistant to synapse elimination. Third, prespecified axons might contact unspecified muscle fibers at random, then use transsynaptic regulatory mechanisms to endow them with appropriate specializations. Fourth, the critical interactions might be with extramuscular guidance cues that would guide axons to appropriate targets, regardless of the target cell's specific molecular identity. See text for evidence that all four mechanisms may play roles in vivo. Finally, not shown here, is a variant in which initially unspecified axons are diversified following contact with prespecified muscle cells.

activity induce qualitative changes in expression of genes that encode fiber type-specific isoforms of contractile proteins (reviewed in Schiaffino & Reggiani 1996; Pette & Staron 1997).

Although mechanisms of guidance and conversion can account for much of neuromuscular specificity, there is compelling evidence that target recognition is also involved. Two cases have been studied in detail. First is fiber type matching. Although conversion plays an important role, fast and slow myotubes actually arise from distinct, committed myoblast populations and express fiber type–specific characteristics independent of innervation (reviewed in Stockdale 1992; Donoghue & Sanes 1994). Thus, fiber type depends on lineage as well as innervation (DiMario & Stockdale 1997), so if fiber type matching occurred strictly by conversion, considerable mismatching should be detectable early in development. In fact, motor units are substantially homogeneous even prior to the period of synapse elimination, indicating that fast and slow motor axons recognize differences between fast and slow myotubes (Thompson et al 1990). More direct evidence for this conclusion comes from the demonstration that reinnervation of intermingled twitch and tonic fibers in frog muscles is highly fiber type–specific (Elizalde et al 1983).

A second phenomenon that involves synaptic selectively is the matching of motor axons and muscle fibers on the basis of their axial position. Motor pools are systematically mapped onto the surface of many muscles, with axons derived from rostral-most neurons projecting to the rostral-most part of the muscle and so on (Landau et al 1962, Brown & Booth 1983, Bennett & Lavidis 1984, Laskowski & Sanes 1987, Bennett & Ho 1988). Importantly, positional selectively is also demonstrable during reinnervation or following muscle transplantation, situations in which extramuscular guidance, timing, and access can be excluded as contributors (Wigston & Sanes 1982, Wigston 1986, Hardman & Brown 1987, Wigston & Kennedy 1987, Laskowski & Sanes 1988). Thus, molecular cues must exist that bias synapse formation between axons and muscle fibers in favor of positionally-matched partners. Muscle cells bear a cell-autonomous, heritable memory of their rostrocaudal position of origin, which might underlie the differences among them that axons recognize (Donoghue et al 1992).

The recognition processes that underlie positional and fiber type matching could act to favor the initial formation of appropriate synapses and/or the elimination of inappropriate synapses. For positional matching, there is evidence for both possibilities: Synaptogenesis appears to be selective from the outset (Wigston & Sanes 1985, Laskowski & Owens 1994), but may be sharpened by selective elimination (Brown & Booth 1983, Bennett & Lavidis 1984, Bennett & Ho 1988, Donahue & English 1989, English 1990, Laskowski et al 1998a). Likewise, fiber type–selective innervation is apparent at initial stages

but enhanced by selective innervation (Elizalde et al 1983, Gates & Betz 1993, Ridge & Rowlerson 1996).

The molecular cues responsible for selective innervation of muscle fibers are unknown. Although numerous physiological and metabolic differences have been identified that distinguish fast- from slow-twitch muscle fibers and fast from slow motoneurons (Gardiner 1993, Schiaffino & Reggiani 1996), no distinguishing cell surface markers have been described for either cell type. On the other hand, rostral and caudal muscles bear different levels and isoforms of ephrins (Donoghue et al 1996), ligands that have recently been implicated in the generation of retinotopic maps (reviewed in Flanagan & Vanderhaegen 1998). Moreover, ectopic expression of ephrins in muscles of transgenic mice impairs formation of rostrocaudal mapping within muscles without detectably perturbing the structure or function of NMJs (Laskowski et al 1998b). This result raises the intriguing possibility that central and peripheral topographic maps make use of similar molecular machinery. In addition, it supports the ideas that different gene products regulate the mechanics and selectivity of NMJ formation.

GENERALIZATIONS ABOUT SYNAPSE FORMATION (FIGURE 11)

Some time ago, one of us referred to the NMJ as "the *E. coli* of synapses," drawing attention to the ways in which the simplicity and accessibility of this synapse have made it an attractive subject for studies of synaptic structure, function, and development (Hall & Sanes 1993). Such studies are often based on the premise that lessons learned at the NMJ will be readily applicable to neuron-neuron synapses. There is, however, a more sinister interpretation of the metaphor: The genome of *E. coli* is so specialized, highly evolved, and limited that it lacks some features of the eukaryotic genome, such as introns. Likewise, the NMJ is a "fail-safe" synapse, relatively incapable of integrating information, and might lack critical features of CNS synapses. Which view is closer to the truth?

NMJs and neuron-neuron synapses are encouragingly similar in ultrastructure and function. In both, nerve terminals adhere to a specialized postsynaptic apparatus and are capped by processes of a glial cell. Both motor and central nerve terminals bear concentrations of synaptic vesicles focused at release sites; the same vesicle proteins are present in both, and mechanisms of vesicular, quantal release are shared (Calakos & Scheller 1996). The postsynaptic membranes are thickened and bear high concentrations of transmitter receptors, sandwiched between a specialized cytoskeletal apparatus and amorphous synaptic cleft material. In many cases (neuronal nicotinic, glycine, and GABA-A receptors, for example) the receptors themselves are homologues of muscle AChRs.

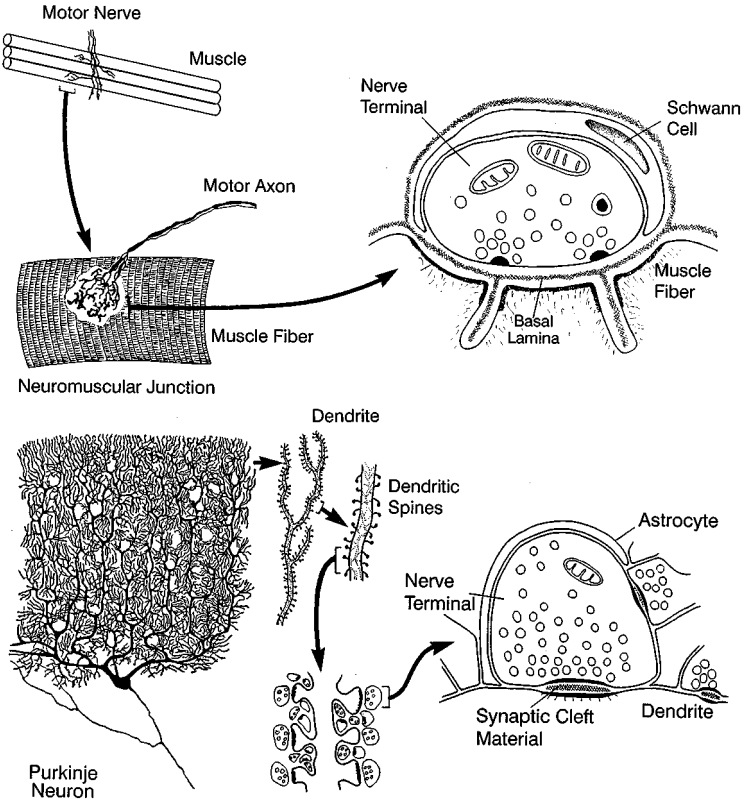

Figure 11 Comparison of the NMJ and central synapses. (*Left*) The distribution of synapses on target cells differs markedly between nerve-muscle and nerve-nerve synapses. In the former, each postsynaptic cell receives a single massive input, whereas in the latter, multiple smaller inputs generally converge on a single target. (*Right*) On the other hand, individual boutons are similar in both synaptic types: The vesicle-laden nerve terminal is capped by glial processes and separated by a cleft containing extracellular material from a thickened, postsynaptic membrane. (Left panels are redrawn from Cajal 1928; right panels are from Sanes & Scheller 1997.)

In addition, several features of neuromuscular and central synaptic development are strikingly similar. Both begin as unspecialized contacts between growth cones and target cells, then differentiate in a series of steps (Vaughn 1989). Neurotransmitter receptors are initially distributed diffusely on dendritic surfaces, then clustered by nerve terminals (Rao et al 1998). Moreover, some proteins are synthesized locally at synaptic sites in dendrites (Gao 1998); synthesis of some central receptors is induced by neuregulins (Ozaki et al 1997); and electrical activity regulates receptor levels via a calcium-dependent pathway

(DeKoninck & Cooper 1995). The γ- to ε-AChR subunit switch documented at the NMJ is paralleled by similar isoform switches in glutamate, GABA, and glycine receptors at synapses in the brain (see Missias et al 1997 for references). At a later stage, synapse elimination occurs on neurons in the brain and peripheral ganglia (Purves & Lichtman 1980, Lohof et al 1996) as it does at the NMJ. In each of these systems, there is reason to believe that naturally occurring reductions in axonal input result from competition between axons innervating the same postsynaptic cell. Finally, although little is known about the factors that account for selective target recognition in either system, it is intriguing that ephrins have been implicated in both (Flanagan & Vanderhaeghen 1998, Laskowski et al 1998b).

On the other hand, the synaptic cleft differs dramatically between the two synaptic types. NMJs are unique among synapses in having a basal lamina extend between their pre- and postsynaptic membranes. Central clefts, in contrast, bear neither a formed basal lamina nor detectable levels of basal lamina components such as laminins or collagens IV. It may be, therefore, that mechanisms of synaptic adhesion differ fundamentally between the two types of synapses: Membrane-matrix interactions mediated by laminins, integrins, and dystroglycan may predominate at the NMJ, whereas cell-cell adhesion molecules such as cadherins may play a predominant role in brain (Colman 1997).

Differences in adhesive mechanism almost certainly imply differences in the signals that mediate target recognition and synaptic maintenance. For example, the stability of the neuromuscular postsynaptic apparatus following denervation, and the recognition of synaptic sites by regenerating axons, may result from the persistence of specializations in the synaptic basal lamina. In contrast, there is little evidence that postsynaptic sites remain specialized or recognizable following denervation of neurons (Purves & Lichtman 1987). In addition, some of the signaling pathways described at the NMJ may function differently or not at all in brain. Agrin is abundant in central neurons and peripheral ganglia (Bowe & Tallon 1995) and is concentrated at some central synapses (Mann & Kroger 1996), but initial attempts to detect defects at hippocampal synapses in agrin-deficient mutant mice have been fruitless (Serpinskaya et al 1997). Also, levels of MuSK and rapsyn are extremely low in brain, and neuronal nicotinic AChRs cluster normally in rapsyn$^{-/-}$ mutant (Feng et al 1998). Nonetheless, nerve-induced receptor aggregation has been clearly described in central neurons, and several proteins have been described that cluster central transmitter receptors and couple them to the cytoskeleton (Kirsch et al 1996). It is therefore reasonable to imagine that central neurons will use aggregating mechanisms similar to those of the NMJ, but different specific molecules.

Even more fundamental may be differences that are evident at the cellular rather than the subcellular level. Muscle fibers receive a single massive input

at a single site, and most of their surface is nonsynaptic. Many central neurons, in contrast, receive numerous small inputs that cover the majority of their dendritic and somatic surfaces. In a sense, the adult central neuron is more akin to a neonatal endplate than to an adult muscle fiber. Even at birth, though, all of the inputs at an NMJ are of a single type, whereas central neurons integrate qualitatively different inputs and control clustering of distinct receptor types at discrete sites. One possibility is that they do so by utilizing a set of parallel pathways, each containing functional homologues of molecules like agrin, MuSK, rapsyn, neuregulin, laminin, and so on. On the other hand, it remains possible that mechanisms used by cells that need to select and deal with only a single input are fundamentally different from those required to deal with multiple types of inputs.

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