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Intercellular Communication That Mediates Formation of the Neuromuscular Junction

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Abstract

Reciprocal signals between the motor axon and myofiber induce structural and functional differentiation in the developing neuromuscular junction (NMJ). Elevation of presynaptic acetylcholine (ACh) release on nerve–muscle contact and the correlated increase in axonal-free calcium are triggered by unidentified membrane molecules. Restriction of axon growth to the developing NMJ and formation of active zones for ACh release in the presynaptic terminal may be induced by molecules in the synaptic basal lamina, such as S-laminin, heparin binding growth factors, and agrin. Acetylcholine receptor (AChR) synthesis by muscle cells may be increased by calcitonin gene-related peptide (CGRP), ascorbic acid, and AChR-inducing activity (ARIA)/heregulin, which is the best-established regulator. Heparin binding growth factors, proteases, adhesion molecules, and agrin all may be involved in the induction of AChR redistribution to form postsynaptic-like aggregates. However, the strongest case has been made for agrin's involvement. "Knockout" experiments have implicated agrin as a primary anterograde signal for postsynaptic differentiation and muscle-specific kinase (MuSK), as a putative agrin receptor. It is likely that both presynaptic and postsynaptic differentiation are induced by multiple molecular signals. Future research should reveal the physiological roles of different molecules, their interactions, and the identity of other molecular participants.

Index Entries: Neuromuscular junction; synapse development; acetylcholine receptor; nerve terminal; receptor tyrosine kinases; extracellular matrix; cell adhesion; growth factors; agrin; proteolysis.

Introduction

Efficient neurotransmission at chemical synapses requires the assembly of a presynaptic terminal containing the machinery for evoked release of neurotransmitters and an apposed postsynaptic membrane containing a high density of the corresponding neurotransmitter receptors. Much of our knowledge about the development and function of chemical synapses has come from studies of a highly developed cholinergic synapse, the vertebrate neuromuscular junction (NMJ). These studies have been facilitated by the accessibility of the NMJ,

the discovery of snake toxins that bind the nicotinic acetylcholine receptor (AChR) with high affinity, and the enormous enrichment of NMJ molecules in marine ray and eel electric organs (which are essentially stacks of modified NMJs). Complementing these tools, special features of invertebrate nervous systems, such as the large size of identified nerve and muscle cells and the relative ease of genetic analysis, have more recently been exploited to increase our understanding of the molecular basis for specificity in NMJ formation (Vactor et al., 1993; Haydon and Zoran, 1994; Bate and Broadie, 1995).

Although the development of the NMJ has been studied for more than four decades, it is only recently that we have gained some insight into the molecular mechanisms that are responsible for inducing and executing the assembly of the synaptic apparatus. In fact, much about these inductive processes remains unknown or controversial. A few general reviews of NMJ structure and development (Hall and Sanes, 1993; Grinnell, 1995) as well as a number of reviews on particular aspects have been published in the last few years. The present article will first briefly review the critical changes in the motor axon and developing myofiber (myotube) that are induced by anterograde (nerve to muscle) and retrograde (muscle to nerve) signals early in NMJ development. Then evidence that specific molecules trigger these local inductive processes will be summarized and updated. Finally, some of the still unanswered questions about the identity of the participating molecules and their possible interactions will be discussed.

Early Inductive Events in NMJ Formation

Functional synaptic transmission occurs within the first day of contact between motor axons and newly formed muscle fibers in rat embryos (Bennett and Pettigrew, 1974; Dennis et al., 1981) and within a few hours of contact in *Xenopus* embryos (Kullberg et al., 1977). In vitro, the earliest responses of the motor axon to a muscle cell can occur within a few minutes after initial contact. ACh release is detected immediately after spontaneous nerve–muscle contact (Kidokoro and Yeh, 1982). In both *Xenopus* and chick nerve–muscle cocultures, there is a low level of spontaneous ACh release from growth cones prior to contact with muscle cells (Hume et al., 1983; Young and Poo, 1983). However, experiments in which *Xenopus* myocytes were manipulated into direct contact with neuron growth cones have shown a dramatic increase in this spontaneous release (Xie and Poo, 1986), indicating a retrograde inductive effect of muscle on the nascent presynaptic terminal (Dan and Poo, 1994).

The earliest well-characterized response of the muscle cell to neuronal contact takes place over a time scale of tens of minutes to hours. This is the accumulation (aggregation) of nicotinic AChRs at sites of contact with the motor axon. Receptor aggregation occurs largely as the result of the redistribution of diffusely organized AChRs (Anderson and Cohen, 1977; Daniels et al., 1987; Hall and Sanes, 1993), but may also involve the local insertion of newly synthesized receptors, as demonstrated in chick nerve–muscle cell cultures (Role et al., 1985; Dubinsky et al., 1989). AChR aggregation is correlated with the formation of a transmembrane complex that spans the submembrane cytoskeleton, the plasma membrane, and the extracellular matrix (ECM), and contains a number of molecules specific to the NMJ (Froehner, 1991; Hall and Sanes, 1993; Gautam et al., 1995). This transmembrane complex appears to be involved in the concentration of AChRs and other functional synaptic components. The spatial and temporal patterns of accumulation of certain molecules in this complex, such as the membrane-cytoskeletal protein rapsyn, are tightly correlated with AChR aggregation. This and other evidence indicates direct involvement of these molecules in receptor accumulation (Froehner, 1991; Gautam et al., 1995). In contrast, accumulation of other components, such as acetylcholinesterase, occurs with a significant delay (Ishikawa and Shimada, 1982;

Chiu and Sanes, 1984), probably because their accumulation requires the prior deposition of other molecules.

For the purposes of this article, the last phase of early inductive interactions is considered to occur more than a day after initial nerve–muscle contact. This phase is characterized by continuing differentiation of the postsynaptic apparatus, the restriction of axon growth to the vicinity of the NMJ, and differentiation of the presynaptic terminal as seen in the formation of active zones for vesicular exocytosis of ACh (Kelly and Zacks, 1969; Kullberg et al., 1977; Hall and Sanes, 1993). Active zones are seen by ultrastructural methods as sites where synaptic vesicles cluster near dense specializations on the cytoplasmic side of the presynaptic membrane. They contain concentrations of voltage-sensitive calcium channels and other molecules critically involved in exocytosis (Cohen et al., 1991; Hall and Sanes, 1993; Kelly, 1993; Robitaille et al., 1993). Studies that directly correlate electrophysiology with ultrastructure have shown active zones to be sites at which vesicles in fact open to the synaptic cleft during quantal release of acetylcholine (Heuser et al., 1979). However, little is known about the interrelationships between the molecular components and how they are assembled.

The Earliest Retrograde Signaling—ACh Release and the Rise in Presynaptic Resting Calcium Levels

The existence of an early retrograde signal from muscle to nerve has been suggested by the observation that the local rate of spontaneous release can be elevated dramatically within seconds by manipulating a myocyte into contact with the growth cone, axon, or even the cell body (Chow and Poo, 1985; Xie and Poo, 1986; Dai and Peng, 1993). This presynaptic response appears to be specific to molecules on the myocyte surface, since only a transient response is elicited by contact with another neuron from the same culture or simple contact with a glass pipet. Furthermore, the response can be elicited by contact with an isolated fragment of muscle cell membrane, suggesting that it is mediated by cell-surface molecules. *Helisoma* (freshwater snail) motor neurons show an increase in evoked neurotransmitter release occurring hours after contact with an appropriate target muscle, but not after contact with an inappropriate target, such as a neuron with which the motoneuron forms an electrical synapse (Zoran et al., 1990, 1991). The delay in this response is believed to correspond to the time needed to assemble the apparatus for excitation–secretion coupling.

Relatively little is known about the molecular basis for the elevation in neurotransmitter release seen after nerve–muscle contact in *Xenopus*. Increased neurotransmitter release is correlated with a rapid, stable increase of two- to threefold in the resting level of free calcium in the growth cone and subsequently in the axon and cell body (Dai and Peng, 1993). Such an increase also occurs when an axon contacts a myocyte during outgrowth. A similar, rapid effect on the calcium "set-point," probably mediated by regulation of intracellular calcium stores, has been observed in cultures of *Helisoma* motoneurons and muscle cells (Zoran et al., 1993). It is followed shortly by a protein kinase A-dependent increase in the calcium influx evoked by depolarization (Funte and Haydon, 1993). The elevation in calcium set point is specific to contact with muscle, since it does not occur with other cell types in *Xenopus* or, in the case of *Helisoma*, with a neuron that forms an electrical synapse with the motoneuron. As with the increase in presynaptic neurotransmitter release, these findings suggest that the presynaptic calcium responses are triggered by specific molecules and their receptors on the surfaces of the muscle and nerve cells, respectively. The cell-surface molecules that mediate these early axonal responses have not been identified, but the observation that membranes prepared from the target muscle can induce a similar elevation in the calcium set point and that this activity of the membranes is trypsin-sensitive (Zoran et al., 1993) suggests

that this retrograde signaling may be mediated by a protein or proteins bound to the muscle cell surface. In this regard, it has been reported that antibodies to NCAM inhibit the stimulation of ACh release by muscle cell contact in *Xenopus* nerve–muscle cultures (Chow, 1990). However, it is not known whether NCAM simply serves here to enhance contact between nerve and muscle or is more directly involved in a signal transduction process (discussed below).

Yet another mechanism by which nerve and muscle cells might communicate when placed into direct contact is through gap junctions. This possibility is supported by the existence of transient and infrequent electrotonic coupling between chick spinal cord neurons and myotubes during synaptogenesis in culture (Fischbach, 1972), by similar observations of electrotonic coupling in *Xenopus* myocytes and spinal cord neurons manipulated into direct contact (Chow, 1990), and by the transfer of Lucifer yellow or tritiated uridine between these two cell types at spontaneously formed *Xenopus* contacts (Allen and Warner, 1991). Structures resembling small gap junctional plaques have been detected by freeze-fracture in the *Xenopus* cocultures (Peng et al., 1980) and loading the *Xenopus* myocytes or neurons with antibodies to gap junction proteins strongly inhibits dye transfer (Allen and Warner, 1991), indicating that the dye transfer is, in fact, mediated by gap junctions. Interestingly, gap junction antibodies did not affect the formation of AChR clusters. Thus, the signaling function of gap junctions during synaptogenesis, if any, probably does not involve postsynaptic membrane differentiation.

The Earliest Anterograde Signaling–Formation of AChR Aggregates and the Postsynaptic Cell Membrane Complex

The discovery that spinal cord neurons induce AChR aggregation at sites of synaptic contact with muscle cells in culture (Anderson et al., 1977; Frank and Fischbach, 1979) led to a search for the molecular signals involved in the induction of AChR accumulation at the NMJ. This search has been complicated by the fact that both the mononucleated myocytes of amphibian embryos and multinucleated myotubes of mammalian and chick embryo muscle cell cultures can be induced to form AChR aggregates by a variety of focally or diffusely applied stimuli, several of which are clearly nonphysiological, but which may activate common signaling pathways and induce the formation of similar, if not identical, transmembrane complexes. I will discuss here candidate molecules and mechanisms that have been shown either to increase the synthesis and local insertion of AChRs or to induce the aggregation of AChRs that already exist in the plasma membrane, thus possibly contributing to the junctional accumulation of receptors.

Regulators of AChR Synthesis

Three molecules have been shown to increase the synthesis of AChR subunits in muscle cells through a specific increase in mRNA levels. These are ascorbic acid, calcitonin gene-related peptide (CGRP), and AChR-inducing activity (ARIA)/heregulin. Of these three, ascorbic acid also has AChR-aggregating activity that may be independent of the regulation of synthesis. This will be discussed in a subsequent section.

Ascorbic acid has been identified as a component of fetal brain extract that increases AChR synthesis by two- to threefold in myotubes of the L5 muscle cell line (Knaack and Podleski, 1985; Knaack et al., 1986). This effect occurs at levels of a few micrograms per milliliter consistent with concentrations of hundreds of micrograms per milliliter in extracts of fetal brain tissue and much lower, although significant, concentrations in serum (Horning, 1975). It is noteworthy also that ascorbic acid release can be evoked from cortical synaptosomes (Milby et al., 1981) and striatal homogenates (Bigelow et al., 1984) in a manner suggestive of synaptic release. Ascorbic acid increases mRNA

levels for the α-subunit of AChR in L5 and primary rat myotubes (Horowitz et al., 1989a,b), although net translation of the message is not correspondingly increased in primary rat myotubes. Thus, in L5 myotubes, the increase in AChR aggregation in the presence of ascorbate may be owing in part to increased AChR synthesis (hence a larger pool of AChRs for aggregation). It remains to be determined whether ascorbic acid plays a similar role during synaptic development in vivo.

CGRP is an alternatively spliced peptide product of the calcitonin gene locus that is found (among other sites in the central [CNS] and peripheral nervous system [PNS]) in motoneurons of the spinal cord and brain (Takami et al., 1985a,b; New and Mudge, 1986; Fontaine et al., 1986) and in their nerve endings at the NMJ (Takami et al., 1985a; New and Mudge, 1986). It is cosecreted with ACh in cultured rat trigeminal ganglion cells (Mason et al., 1984) and at the NMJ (Uchida et al., 1970). In chick embryo myotube cultures, CGRP moderately upregulates AChR synthesis in correlation with a threefold increase in the level of α-subunit mRNA. This increase appears to be mediated by an increase in intracellular cAMP (Fontaine et al., 1987; Laufer and Changeux, 1987). It has thus been suggested that CGRP contributes to the upregulation of surface AChR at the developing NMJ and in the adult. The reported patterns of CGRP expression during embryonic and postnatal development are consistent with a role for this peptide in NMJ development, but possibly not at the earliest stages or in all junctions. In chick embryos, CGRP has been detected in motoneurons at the earliest stages of NMJ formation, but only in a subpopulation of those neurons and neuron terminals (New and Mudge, 1986). In *Xenopus* embryos and larvae, CGRP immunoreactivity is found at the myotomal NMJs only after stage 32, in correlation with the accumulation of synaptic vesicle markers in presynaptic specializations, whereas junctional AChR clusters appear as early as stage 22 (Peng et al., 1989). Thus, if CGRP is involved in the early stages of NMJ development in *Xenopus*, it must be present and active at very low levels.

ARIA is a member of a family of ligands for the Neu family of receptor tyrosine kinases (Falls et al., 1993; Fischbach et al., 1994). These ligands, discovered independently by different approaches (Holmes et al., 1992; Wen et al., 1992; Marchionni et al., 1993), are alternatively spliced products of a single gene and are also known as heregulins, Neu differentiation factors (NDF), and glial growth factors (GGF). ARIA was purified by Fischbach and colleagues from chick embryo brain as a 42-kDa AChR, inducing activity that selectively increases the rate of insertion of newly synthesized AChR into the plasma membrane of chick embryo myotubes three- to fivefold (Usdin and Fischbach, 1986; Fischbach et al., 1994). As with ascorbic acid and CGRP, this upregulation is correlated with a selective increase in α-subunit mRNA levels. In mouse myotubes, however, ARIA induces a 10-fold increase in levels of the AChR ε-subunit mRNA as well as a twofold increase in γ, δ-, and α-subunit mRNAs and a modest 20–50% increase in the rate of new receptor insertion (Martinou et al., 1991). The marked upregulation of the ε-subunit mRNA suggests a possible role in the replacement of embryonic AChRs with the adult form that occurs postnatally (Hall and Sanes, 1993). The effect of ARIA on mRNA levels in mouse myotubes is not mimicked by CGRP or other treatments that elevate intracellular cAMP, suggesting that a different signal transduction mechanism is involved. In fact, upregulation of AChR expression by ARIA is coupled to the tyrosine phosphorylation of a 185-kDa membrane protein(s) of the Neu family (Corfas et al., 1993), probably a complex of the erbB2 and erbB3 receptor tyrosine kinases (Jo et al., 1995; Altiok et al., 1995). This upregulation also requires activation of the Ras/MAP kinase signal transduction pathway and the phosphatidylinositol 3-kinase pathway, downstream of erbB receptor activation (Tansey et al., 1996; Si et al., 1996).

ARIA mRNA expression is found at various sites in the chick and mouse CNS, including

high levels in motoneurons of the brain and spinal cord. Concentrated expression in spinal motoneurons is first detected as early as embryonic d 5 (E5) in the chick, around the time when the first synaptic contacts with muscle are formed. ARIA has been detected immunocytochemically in the adult mouse NMJ, where it is associated with the junctional basal lamina (Jo et al., 1995). This is consistent with the ability of the junctional basal lamina to induce synapse-specific AChR subunit expression in regenerating muscle fibers in the absence of innervation (Goldman et al., 1991; Brenner et al., 1992; Jo and Burden, 1992). ARIA has also been detected by E16 in the chick NMJ, where it has been localized to the presynaptic side of the junctional basal lamina by immunogold labeling (Goodearl et al., 1995). However, it has not been detected at earlier stages when synaptic accumulations of AChR are present. Proteolytic fragments of ARIA containing the EGF-like domain, but not the heparin binding immunoglobulin-like domain, are active, and similar fragments are released from cerebellar neurons by limited proteolysis. Thus, it has been suggested that ARIA deposited in the ECM is released in active form by endogenous protease activity (Loeb and Fischbach, 1995). The extracellular matrix-bound form is presumably released from the presynaptic cell membrane by cleavage of membrane-bound pro-ARIA (Falls et al., 1993).

Adding to the complexity of evaluating the developmental role of ARIA at the NMJ is a recent report (Moscoso et al., 1995) showing that ARIA is expressed by mammalian muscle *in situ* and in vitro, as are the erbB2 and erbB3 receptor tyrosine kinases. Interestingly, these receptors are first detected immunocytochemically at E18 and become localized to the NMJ perinatally, somewhat in advance of the junctional localization of ARIA. This junctional localization of the ARIA receptors appears to be tied to the overall development of the postsynaptic membrane complex, since it does not occur in rapsyn knockout mice, which fail to form synaptic AChR aggregates.

To summarize, the in vitro activity and the in vivo patterns of gene expression and localization of ARIA are consistent with a role in regulating AChR synthesis at the developing NMJ. In this respect, the strongest evidence argues for a role during the perinatal period (in mammals) when junctional AChR expression is sustained in the face of activity-dependent downregulation in the rest of the muscle fiber (Hall and Sanes, 1993). As with ascorbate and CGRP, the definition of ARIA's physiological role awaits definitive experiments.

Inducers of AChR Aggregation

Agrin

The discovery of high-mol-wt AChR-aggregating activity in conditioned media from neuroblastoma-glioma cell lines (Christian et al., 1978), embryonic brain extracts (Podleski et al., 1978; Jessell et al., 1979), and primary neuron cultures (Schaffner and Daniels, 1982) that was independent of new receptor synthesis (Christian et al., 1978; Schaffner and Daniels, 1982) led to the hypothesis that motoneurons secrete a macromolecule (protein) that induces postsynaptic AChR aggregation at the developing NMJ (reviewed in Daniels et al., 1987). Subsequent to the initial reports on AChR-aggregating activities, it was demonstrated that a signal that can direct postsynaptic differentiation in regenerating muscle fibers in the absence of nerve resides in the synaptic basal lamina at the NMJ in *Rana* (Burden et al., 1979). This suggested the possibility that a neuronally secreted "aggregation factor" was deposited in the synaptic basal lamina.

The best candidate for such a neuronal AChR aggregation factor, and the only one thus far to be purified and sequenced, is agrin (McMahan, 1990), a proteoglycan of about 400 kDa (Tsen et al., 1995; Penzer et al., 1995) originally isolated from the ECM of *Torpedo* electric organ (Godfrey et al., 1984; Nitkin et al., 1982). Since agrin and its putative receptor have been the subject of recent reviews (McMahan et al., 1992; Rupp et al., 1992; Bowe and Fallon, 1995; Kleinman and Reichardt, 1996), I will only

attempt to summarize the evidence with respect to the physiological role of agrin and its receptor(s) in postsynaptic AChR aggregation. Agrin was isolated on the basis of its ability to induce the formation of AChR aggregates on the surface of chick myotubes (Godfrey et al., 1984; Nitkin et al., 1987). The agrin gene has been cloned (Rupp et al., 1991; Tsim et al., 1992), and it is expressed in motoneurons of chick and rat at stages of embryonic development appropriate for involvement in NMJ formation (Rupp et al., 1991; Tsim et al., 1992). Both agrin-like immunoreactivity and AChR aggregation activity that can be immunoprecipitated with agrin antibodies are found in embryonic chick motoneurons (Magill-Solc and McMahan, 1988). Moreover, messenger RNAs for alternatively spliced isoforms of agrin that have a high level of AChR-aggregating activity in vitro (Ruegg et al., 1992; Ferns et al., 1992, 1993) are expressed preferentially by various neurons, including motoneurons (Ma et al., 1993; Stone and Nikolics, 1995), whereas only the relatively inactive forms are expressed by muscle. Agrin is transported in the anterograde direction in the frog sciatic nerve (Magill-Solc and McMahan, 1990) and is concentrated in the basal lamina at the NMJ (Fallon et al., 1985). Focal exposure of muscle cell surfaces to agrin by contact with transfected CHO cells (Campanelli et al., 1991) or to deposits laid down by motoneurons on an adhesive substrate (Cohen et al., 1995a) results in focal aggregation of AChR, as would be expected for a molecule that induced focal postsynaptic differentiation at the developing NMJ. It has been reported that agrin is consistently deposited very early at the developing NMJ in *Xenopus* nerve–muscle cocultures (Cohen and Godfrey, 1992). However, others have found a more erratic pattern of agrin deposition in the same type of coculture using the same monoclonal antibodies (MAbs) to agrin (Anderson et al., 1995). Based on the overall distribution of agrin immunoreactivity in their experiments, the latter investigators concluded that the agrin revealed by these antibodies in *Xenopus* cocultures is derived largely from nonneuronal epithelial cells and is sometimes, but not consistently, deposited at developing synapses. Considering that soluble agrin is active at approx 10^{-13} *M* (Nitkin et al., 1987), it is possible that the concentrations of agrin necessary to induce AChR aggregation at developing NMJs are below the limits of even the sensitive methods of detection used by the latter investigators. Nevertheless, the apparent discrepancy between the two groups' results remains to be resolved.

The first direct evidence that agrin is required for nerve-induced AChR aggregation was the report that an antiserum against *Torpedo* agrin prevented the aggregation induced by chick motoneurons cocultured with rat myotubes (Reist et al., 1992). This antiserum crossreacted with chicken, but not rat agrin, and had no effect on the aggregation induced by rat motoneurons, consistent with a requirement for neuronal agrin. More recently, it was shown that NMJ formation is grossly disrupted in agrin "knockout" mice that fail to express the neuronal agrin isoform and have a sharply reduced level of expression of muscle agrin (Gautam et al., 1996). The "knockout" mice display a drastic reduction in the number of nerve-associated AChR aggregates, and those few that form are reduced in size and density, both at 15 d (shortly after the first junctional aggregates form) and 18 d *in utero*. These results strongly support the hypothesis that neuronal agrin is an important signal for postsynaptic membrane differentiation. However, they are also consistent with the possibility that one or more other signals may act independently as inducers, although not with the potency of agrin.

The Agrin Receptor

Results from four research groups (Campanelli et al., 1994; Bowe et al., 1994; Gee et al., 1994; Sugiyama et al., 1994) have indicated that agrin binds to α-dystroglycan, a subunit of the dystrophin-associated glycoprotein complex (Ervasti and Campbell, 1991) in striated muscle plasma membranes. In *Xenopus* nerve-muscle cocultures, α-dystroglycan distribution closely

follows that of AChR in developing synapses as well as in spontaneously formed AChR aggregates (Cohen et al., 1995b). In addition, mutant muscle cells that are defective in proteoglycan synthesis and have altered α-dystroglycan are deficient in spontaneous (Gordon et al., 1993) and agrin-induced (Ferns et al., 1993) AChR aggregation as well as agrin binding (Sugiyama et al., 1994). However, these cells aggregate AChRs in response to innervation (Gordon et al., 1993). Attempts to block either the aggregating activity of agrin or nerve-induced aggregation with an MAb against α-dystroglycan have yielded results ranging from strong inhibition to none at all in the hands of different groups (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; Cohen et al., 1995b). Although the muscle isoform of agrin binds α-dystroglycan with a somewhat higher affinity than the neuronal isoform (Sugiyama et al., 1994; Gesemann et al., 1996), it has much lower AChR-aggregating activity (Ferns et al., 1993), and the addition of excess muscle agrin blocks the binding of the neural form to α-dystroglycan, but does not inhibit its aggregating activity (Bowen et al., 1996). Moreover, it has been shown that an active C-terminal fragment of chicken agrin that does not bind α-dystroglycan has AChR aggregating activity, whereas a larger fragment that binds α-dystroglycan has no aggregating activity (Gesemann et al., 1995, 1996; Meier et al., 1996). Rather, AChR-aggregating activity of the fragments was correlated with the ability to induce phosphorylation of the AChR β-subunit (*see* discussion *below*). Thus, although α-dystroglycan is likely to be involved in the formation and maintenance of AChR aggregates and clearly interacts with agrin, its role as a primary mediator of the agrin signal is in doubt.

A newer candidate receptor for agrin (or other signaling molecules from the nerve) is muscle-specific kinase (MuSK), a receptor tyrosine kinase that is expressed almost exclusively by developing skeletal muscle and is concentrated at the NMJ of adult muscle (Valenzuela et al., 1995). Extrajunctional expression of MuSK is downregulated perinatally, and is upregulated as a result of denervation and inactivity of the muscle, as with the AChR. MuSK is highly homologous to a receptor tyrosine kinase cloned from a *Torpedo* electroplax cDNA library that is expressed in the electric organ and skeletal muscle of that species (Jennings et al., 1993), and even more closely related to Nsk2, a receptor tyrosine kinase that is primarily expressed in developing skeletal muscle in mouse (Ganju et al., 1995). The three represent a class of receptor tyrosine kinases with distinct primary structure in their ligand binding extracellular domains. MuSK "knockout" mice show no signs of NMJ formation or AChR aggregation, and consequently die at birth, although skeletal muscle development is not grossly abnormal (DeChiara et al., 1996). Myotubes grown from the muscle of these embryos fail to aggregate AChRs in response to soluble agrin, suggesting that MuSK is an essential part of the signal transduction process leading to AChR aggregation. Moreover, the observation that tyrosine phosphorylation of MuSK occurs within 1 min after exposure of myotubes to the neuronal, but not to the inactive muscle isoform of agrin, suggests that it acts at or near the initiation of signal transduction (Glass et al., 1996). Although binding of agrin to the isolated ectodomain of MuSK has not been detected in vitro, crosslinking experiments have shown that agrin and MuSK form a molecular complex when an active fragment of agrin is added to myotubes, but not myoblasts. In addition, a soluble fusion protein containing the ectodomain of MuSK shows increased binding to myotube, but not myoblast surfaces in the presence of agrin. Similarly, phosphorylation of MuSK in the presence of agrin occurs only in differentiated myotubes. These results suggest that agrin binding to and activation of MuSK are mediated by an accessory molecule that is expressed after myoblast fusion (Glass et al., 1996). A recent study using transfected fibroblasts indicates that MuSK can be clustered, immobilized, and activated by rapsyn, and that the transfected MuSK phosphorylates AChR β-subunits. This suggests a role for rapsyn in the synaptic localization of MuSK

and possibly in upregulating MuSK's phosphorylation activity at the NMJ (Gillespie et al., 1996). It would be interesting to determine if the synaptic localization of MuSK is eliminated in rapsyn "knockout" mice, as is the case for the synaptic localization of ARIA receptors (Moscoso et al., 1995).

Growth Factors as Inducers of AChR Aggregation

Growth factors isolated on the basis of their ability to promote the proliferation of various cell types have since been shown to promote the survival or differentiation of postmitotic cells, such as neurons or muscle (Rifkin and Moscatelli, 1989; Schlessinger and Ullrich, 1992). Peng and colleagues (1991) have shown that basic fibroblast growth factor (FGFb) can induce focal AChR aggregation on *Xenopus* myocytes when it is presented on the surface of polymer microbeads. This effect appears to be specific, since beads coated with certain other growth factors, denatured FGFb, or proteins of similar charge had no effect. However, similar effects were observed with bead-applied IGF-I and insulin, both of which act through receptor tyrosine kinases. These investigators have also shown that the action of FGFb-coated beads is inhibited by suramin, a blocker of the activity of various heparin binding growth factors, as well as by inhibitors of protein tyrosine kinases, as would be expected for an effect mediated by FGFb or a similar growth factor and its receptor. Previously, Peng and colleagues had shown that uncoated polymer beads (not quenched with serum as in the experiments cited above) can induce AChR aggregation (Peng et al., 1981). The observation that the AChR-aggregating effect of uncoated polystyrene beads can be inhibited by the same substances as the FGFb-coated beads suggests that the uncoated beads may somehow present endogenous growth factors to their receptors on the myocyte surface (Baker et al., 1992). These results suggest that the axon provides a signal to release a matrix-bound growth factor, allowing it to bind its receptor. This is consistent with the observation that *Xenopus* embryonic myotomal muscle expresses FGFb and its receptor during the time of synaptogenesis. FGFb is also associated with the ECM of developing rat muscle (Gonzales et al., 1990). However, only a low level of FGFb immunoreactivity has been detected on *Xenopus* muscle cells in culture (Baker et al., 1992).

Peng and colleagues have demonstrated effects similar to those of FGFb with beads coated with a novel heparin binding growth-associated molecule (HB-GAM). They also found that the bath application of HB-GAM inhibited nerve-induced AChR aggregation, presumably by masking the positional information provided by the nerve (Peng et al., 1995). Although only 20% of beads coated with the most active form of agrin induced AChR aggregation on *Xenopus* myocytes, 80% of beads coated with agrin and then HB-GAM induced aggregation (Daggett et al., 1996). Moreover, HB-GAM binds to deposits of agrin laid down on the culture substrate by neurites. Based on these observations, they suggest that agrin may serve to present HB-GAM or another growth factor to its receptor on myocytes (Daggett et al., 1996). HB-GAM is robustly expressed on the surface of *Xenopus* myocytes in vivo and in vitro as well as at developing rat neuromuscular junctions in vivo, and becomes concentrated at sites of nerve-induced and spontaneous AChR aggregates in vitro (Peng et al., 1995; Szabat and Rauvala, 1996). These findings make HB-GAM a more likely candidate than FGFb for an anterograde signaling molecule, but there has been no direct demonstration of the involvement of either of these growth factors.

Involvement of Ascorbic Acid in AChR Aggregation

Ascorbic acid has been identified as a component of fetal brain extract that increases the deposition of collagen and basal lamina on rat myotubes in primary culture in correlation with increased AChR aggregation (Kalcheim et al., 1982a). Furthermore, the increases in both ECM deposition and AChR aggregation induced in such cultures by the addition of chick ciliary

ganglion or spinal cord explants are inhibited by ascorbate oxidase (Kalcheim et al., 1982b, c, 1985, Vogel et al., 1987). In agreement with these observations, cis-hydroxyproline, a specific inhibitor of collagen synthesis, prevents the clustering of AChR at nerve–muscle contacts in chick ciliary ganglion–muscle cocultures (Bixby, 1994). Thus, in primary cultures of rat myotubes the effects of ascorbic acid on AChR distribution appear to be tied to its effects on the deposition of ECM molecules, such as collagens. Further experimentation will be required to determine if ascorbic acid plays a specific physiological role in postsynaptic membrane differentiation, but with its multiplicity of actions on muscle and other cell types (Third Conference on Vitamin C, Burns et al., 1987), it is likely that ascorbic acid plays at least a permissive role in NMJ development.

Involvement of Proteases and Their Substrates in the Extracellular Matrix

The models for the molecular basis of nerve-induced AChR aggregation discussed so far have involved the secretion (or cell-surface expression) of neuronal factors that influence AChR expression or distribution by interacting essentially in an additive fashion with components of the muscle cell surface. A novel hypothesis to explain the induction of postsynaptic differentiation by nerve has been proposed and supported by Anderson and colleagues (Anderson, 1986; Anderson et al., 1991; Champaneria et al., 1992). According to this essentially subtractive hypothesis, a specific proteolytic cascade, perhaps analogous to that which occurs in blood clotting, is activated when the motor axon and muscle fiber come into contact. This focal proteolytic activity serves to remove existing ECM molecules from the muscle cell surface, activating their receptors (integrins) and triggering the redistribution and *de novo* deposition of the molecules that form the postsynaptic transmembrane complex. It is, in fact, well established that growing axons secrete proteolytic activities that digest molecules in the culture substrate (Krystosek and Seeds, 1984; Pitmann, 1985). Furthermore, immunohistochemical and zymographic observations in vivo as well as experiments with protease inhibitors in vivo and in vitro have implicated proteases (including those of the thrombogenic and fibrinolytic pathways) in the activity-dependent elimination of synapses between nerve and muscle (O'Brien et al., 1984; Connold et al., 1986; Festoff et al., 1990; Liu et al., 1994a, b; Zoubine et al., 1996).

Anderson and colleagues have provided three lines of evidence to support their focal proteolysis model for the initiation of synaptogenesis. First, at an early stage in the formation of the NMJ in *Xenopus* nerve–muscle cocultures, a swath of heparan sulfate proteoglycan (HSPG) is removed from the myocyte surface, prior to the *de novo* deposition of muscle HSPG at sites of AChR aggregation (Anderson, 1986; Swenarchuk et al., 1990). This removal of HSPG appears to be specific to neurite–myocyte contacts that are sites of synaptogenesis (Anderson, 1986). Second, a gelatinase activity (as defined by the removal of fluorescently labeled gelatin from the underlying substrate) is concentrated at sites of synaptic development (Champaneria et al., 1992). Finally, the AChR aggregation ability of differently composed polymer beads with surfaces altered in different ways is directly correlated with their ability to desorb fluorescently labeled proteins from a glass coverslip rather than with their charge or their ability to adhere to the myocyte. This desorptive ability would then mimic proteolytic activity in removing ECM molecules from the myocyte surface (Anderson et al., 1991). Thus, there is considerable evidence supporting the involvement of proteolytic activity in the induction of postsynaptic differentiation. It will now be important to identify the specific proteases acting at the developing synapse and to carry out experiments to test their roles directly.

Cell–Cell Adhesion as a Signal for Postsynaptic Differentiation

At the earliest stages of NMJ formation *in situ* and in culture, membranes of innervating motor axons make close contact with the muscle cell

surface, occasionally forming junctions resembling puncta adherens (Hirano, 1967; Kelly and Zacks, 1969; James and Tresman, 1969; Shimada et al., 1969; Nakajima et al., 1980; Takahashi et al., 1987). An adhesive interaction between the axon and myotube is also suggested by the observation that axons in nerve–muscle cultures are partially or completely surrounded by intimately contacting myotube surfaces (James and Tresman, 1969) adjacent to sites of axon-induced AChR aggregation (Dutton et al., 1995).

Some nonphysiological stimuli that induce AChR aggregation also involve adhesion (Bloch and Pumplin, 1988). One example that has been studied in great detail is the aggregation of AChR at sites where myotubes adhere to the culture substrate (Axelrod et al., 1976; Bloch and Geiger, 1980). Within these patches, membrane domains with a high density of AChR interdigitate with adhesion domains resembling the focal adhesions found in cultured fibroblasts (Bloch and Pumplin, 1988) and domains rich in clathrin-coated membrane (Pumplin and Bloch, 1990). The mechanism by which the formation of adhesion domains triggers the adjacent aggregation of AChR is not well understood. It has been suggested (Bloch and Pumplin, 1988) that the clathrin-rich domains represent sites of increased insertion of AChR and other membrane components, and that the restricted mobility of these components in the vicinity of actin filament-rich adhesion domains might be sufficient to precipitate the formation of the specialized membrane–cytoskeletal complex that immobilizes the AChRs (Bloch and Pumplin, 1988; Pumplin, 1989; Bloch and Morrow, 1989; Daniels et al., 1990; Bloch et al., 1991). The induction of AChR aggregation by positively charged polymer beads (Peng et al., 1981) that adhere to *Xenopus* myocytes in culture may involve mechanisms similar to those mediating aggregation at sites of cell–substrate adhesion. However, alternative explanations for the effects of beads have been proposed (discussed in the previous sections). Whatever the mechanisms, the formation of AChR aggregates at bead contact sites is accompanied by the disappearance of aggregates from other sites on the myocyte (Peng, 1986; Champaneria et al., 1992) as occurs during innervation in culture (Anderson and Cohen, 1977), suggesting that the global effect of bead contact on myocyte cell-surface differentiation is similar to that of a physiological stimulus.

If adhesive interactions are actually involved in the induction of AChR aggregation or other early events in NMJ formation, they must be mediated by interacting molecules on the surfaces of developing muscle fibers and axons. Embryonic muscle and spinal cord neurons express forms of the neural cell adhesion molecule NCAM (Grumet et al., 1982; Rutishauser et al., 1983; Covault and Sanes, 1985, 1986; Londmesser et al., 1988) at stages of development early in the formation of NMJs. Furthermore, antibodies to NCAM inhibit the adhesion between chick spinal cord or ciliary ganglion neurons and myotubes in culture (Rutishauser et al., 1983; Bixby and Reichardt, 1987). However, exposure of chick ciliary ganglion neuron-muscle cultures to anti-NCAMs appears to have little effect on NMJ formation as defined by the accumulation of presynaptic vesicle markers opposite sites of AChR aggregation (Bixby and Reichardt, 1987). Embryonic muscle and nerve cells express other adhesion molecules, such as *N*-cadherin (Hatta et al., 1987), which could mediate adhesion by homophilic or heterophilic interactions. On the other hand, some adhesion molecules, such as L1, which are expressed by motoneurons, but not muscle at the early stages of NMJ formation (Sanes et al., 1986), might still be involved in heterophilic interactions between the two cell types (Rutishauser, 1993). The possible roles of these molecules in NMJ formation have not been tested. Adhesive interactions between motor axons and muscle could also be mediated by integrins or cell membrane glycoproteins (for example, extracellular components of the dystrophin-associated glycoprotein complex) binding to extracellular matrix molecules on the nerve or muscle cell surfaces. Integrins have been localized to the neuromuscular

junction and to AChR aggregates of myotubes in culture (Bozyczko et al., 1989; Martin et al., 1991), but there have not, as yet, been any functional tests of the role of integrins in NMJ formation. In addition, although some extracellular matrix components accumulate at NMJs within the first day after functional innervation occurs (Chiu and Sanes, 1984; Anderson et al., 1984; Godfrey et al., 1988), the earliest contacts between motor axons and muscle show little accumulation of ECM as seen by electron microscopy (Hirano, 1967; Kelly and Zacks, 1969; James and Tresman, 1969; Shimada et al., 1969; Nakajima et al., 1980; Takahashi et al., 1987) In addition, the accumulation of laminin, a major basal lamina component and ligand for integrins, appears to lag behind the formation of AChR aggregates at rat NMJs developing in culture (Dutton et al., 1995). Similarly, visible accumulation of laminin and HSPG lags behind AChR aggregation induced by agrin in chick myotube cultures (Nitkin and Rothschild, 1990). Thus, the interactions of cell membrane molecules with certain ECM components may play a more important role in the maturation and stabilization of the NMJ than in its initial formation.

Restriction of Axon Growth and Formation of Active Zones

The concept that local signals extrinsic to the muscle fiber may restrict axon growth to the vicinity of the NMJ and induce presynaptic differentiation was suggested by observations in regenerating NMJs in *Rana* cutaneous pectorus muscles. When both muscle fiber and nerve (including its sheath) are damaged and the muscle fiber is prevented from regenerating, the motor axon grows back to the original site on the persisting muscle basement membrane, where it forms a functional nerve terminal. This regenerated terminal contains active zones that are aligned with the junctions between the basal lamina of the primary synaptic cleft and the secondary folds, that is, the sites opposite which the original active zones were located (Sanes et al., 1978; Glicksman and Sanes, 1983; Kuffler, 1986). An approach similar to the one leading to the discovery of agrin has yielded a preliminary characterization of a smaller protein(s) in *Torpedo* electroplax that induces the formation of axonal varicosities enriched in synaptic vesicle antigens in cultured chick motor neurons (Werle and McMahan, 1991). The authors have named this protein(s) agritin. Aside from this, two molecular components concentrated in the junctional basal lamina have been suggested as inducers of presynaptic differentiation and as "stop signals" for the motoneuron growth cone, largely on the basis of in vitro experiments. In evaluating in vitro experimental findings regarding the role of various molecules in the induction of presynaptic differentiation, it should be kept in mind that axons can be induced to form presynaptic-like accumulations of vesicles close to the membrane by the nonphysiological stimulus of contact with positively charged polymer microbeads (Burry, 1980; Peng et al., 1987). The in vitro evaluation of the role of putative stop signals is also subject to a caution: Axons in nerve–muscle culture systems may extend for hundreds of microns over one or more muscle cells, forming multiple synaptic structures at irregular intervals (for example, (Anderson and Cohen, 1977; Cohen et al., 1987; Role et al., 1987; Dutton et al., 1995). Thus, normal "stop signals" may be lacking or ineffective in culture.

S-Laminin

S-laminin or laminin β2, originally identified as an antigen of the junctional basement membrane (Sanes and Hall, 1979), is a homolog of the β1 chain of laminin, a major glycoprotein of the basal lamina. S-laminin is concentrated in the neuromuscular junction and certain nonmuscle basal laminae, including that of the kidney glomerulous, substituting for the β1 chain (Hunter et al., 1989a, b; Sanes et al., 1990). Expressed fragments of S-laminin containing its three Leu-Arg-Glu sequences are selectively

adhesive for motoneuron-like cells in vitro (Hunter et al., 1989b, 1991). These polypeptides selectively inhibit the neurite outgrowth of motoneuron-like cells and chick spinal motoneurons stimulated by laminin, fibronectin, or collagen I, and the outgrowth of ciliary ganglion neurons is halted at interfaces between laminin and laminin overlayed on the polypeptides (Porter et al., 1995). These findings, together with the observation that *S*-laminin accumulates at the same sites as AChR on cultured muscle cells (Martin et al., 1995), are consistent with the idea that *S*-laminin contributes to the termination of axon outgrowth at the developing NMJ. However, *S*-laminin "knockout" mice do not show excessive axon outgrowth, that is, outside the sites of NMJ formation (Noakes et al., 1995). Conversely, in rapsyn "knockout" mice, which lack postsynaptic AChR aggregates, but do have synaptic accumulations of *S*-laminin, axons do show excessive outgrowth, although presynaptic differentiation is relatively normal (Gautam et al., 1995). Thus, other determinants that accumulate at the developing NMJ may be sufficient to prevent extrajunctional axon extension, and *S*-laminin alone may not be sufficient. This may be explained (Porter et al., 1995) by the presence of Leu-Arg-Glu sequences in other proteins, such as agrin, that are concentrated in the junctional cleft. In, this light, it is interesting that excessive axon outgrowth is found in "knockout" mice that are deficient in postsynaptic differentiation because of deletions for agrin and MuSK as well as rapsyn. At present, there is only fragmentary information on the extent to which ECM components containing the putative stop signal are concentrated at nerve–muscle contacts in these "knockout" mice (*see* Kleinman and Reichardt, 1996). Regarding presynaptic differentiation, the *S*-laminin "knockout" mice demonstrate defective motor nerve terminals that have few active zones and a failure to concentrate synaptic vesicles in the portion of the terminal close to the junction (Noakes et al., 1995), suggesting that *S*-laminin plays an important role in the development of the presynaptic terminal. It remains to be determined whether this effect is secondary to other alterations in the structure of the NMJ, such as the increased encroachment of Schwann cell processes into the junctional cleft. In this respect, it would be interesting to determine the effects of this deletion on differentiation of the NMJ in culture, where the roles of different cell types can be more easily distinguished.

Agrin as a "Stop Signal" and Inducer of Presynaptic Differentiation

It has recently been shown that Chinese hamster ovary cells expressing neural or muscle isoforms of agrin on their surfaces are adhesive for chick ciliary ganglion neurons and that axons grow onto islands of the transfected cells, but not over them (Campagna et al., 1995). This adhesive interaction may be mediated by binding of agrin to NCAM (Tsen et al., 1995; Burg et al., 1995). In addition, contact with the agrin-expressing cells induces focal accumulations of a synaptic vesicle marker protein, synaptotagmin (Campagna et al., 1995). These results suggest a role for the accumulations of agrin at developing synapses in the limitation of axon growth and the differentiation of the presynaptic terminal. As mentioned above, in both the agrin and MuSK "knockout" mice (Gautam et al., 1996; DeChiara et al., 1996) motor axons show extensive ramification over the length of diaphragm muscle fibers instead of terminating in a narrow band lateral to the main nerve trunk. This is consistent with a role for the agrin signaling pathway in the restriction of axon growth, but does not directly address the molecular identity of the "stop signal."

Growth Factors as Inducers of Presynaptic Differentiation

It has been shown that polymer beads coated with FGFb can induce at least two aspects of presynaptic differentiation in *Xenopus* spinal cord neurons in culture (Dai and Peng, 1995, 1996). Extensive accumulation of synaptic

vesicles occurs, particularly along the membrane facing the bead. In addition, the sites of this vesicle accumulation show elevated levels of depolarization-induced calcium uptake, suggesting the local accumulation of voltage-sensitive calcium channels. These effects, as with the postsynaptic effects described above, are inhibited by specific blockers of protein tyrosine kinases and inhibitors of heparin binding growth factors. Further support for the idea that presynaptic differentiation can be induced by growth factors released by muscle or presented on its surface is provided by the observation that functional and structural maturation of the presynaptic neurotransmitter release apparatus can be enhanced by the neurotrophin NT-3 and to a lesser extent BDNF in cultures of *Xenopus* myocytes and spinal neurons (Wang et al., 1995). Both of these neurotrophins are synthesized by muscle cells (Schecterson and Bothwell, 1992; Henderson et al., 1993; Koliatsos et al., 1993). These effects appear to be mediated presynaptically and are sensitive to a drug that specifically inhibits tyrosine phosphorylation by the Trk family of neurotrophin receptors (Wang et al., 1995).

Criteria For Evaluation of the Roles of Putative Inducers of Early Synaptic Differentiation

As discussed above, there are at least a few putative molecular inducers of presynaptic and postsynaptic differentiation in the early stages of NMJ formation, as suggested by their activities in vitro, and by their expression in motoneurons and/or skeletal muscle cells at the appropriate sites and developmental stages. In addition to these molecular species, there is the possible involvement of molecular mechanisms, such as cell–cell adhesion and focal proteolysis, for which the specific molecular candidates have not been named (although many have been demonstrated to be essential for other aspects of cellular differentiation).

Ultimately, the proof that any candidate molecule is directly involved in these inductive processes requires converging lines of evidence that go beyond the demonstration of in vitro activity. This should include demonstrations that the molecule is present at the specific site at or before the time of the inductive event and that the deletion of the molecule, either by specific blockage or genetic manipulation, results in inhibition of differentiation. A hypothesis involving any of these molecules would be further strengthened by the identification of its functional receptor in the cell type involved, of the receptor's presence at the appropriate stage of development, and of an inhibitory effect of the receptor's deletion or blockage. Even in the case of agrin, the most extensively studied and experimentally supported candidate molecule, these lines of evidence are as yet incomplete or subject to some controversy.

Molecular-Level Interactions Contributing to Early Synaptic Differentiation

Although critical evidence supporting or refuting the involvement of a number of molecules in the induction of synaptic development continues to be generated, it is interesting to consider the ways in which different molecules or mechanisms might interact to facilitate induction. Since developing muscle fibers will form AChR aggregates "spontaneously" (Vogel et al., 1972; Sytkowski et al., 1973) and in response to a variety of defined stimuli other than innervation (discussed above), the muscle cell probably has an intrinsic program, activated by these stimuli, for the initial steps of postsynaptic differentiation. Similarly, the axon apparently responds to a variety of stimuli by forming presynaptic-like structures (described above). Thus, it is useful to consider a few of the common pathways through which these stimuli may act. Figure 1A and B indicates some of the possible interactions discussed above and in the following section.

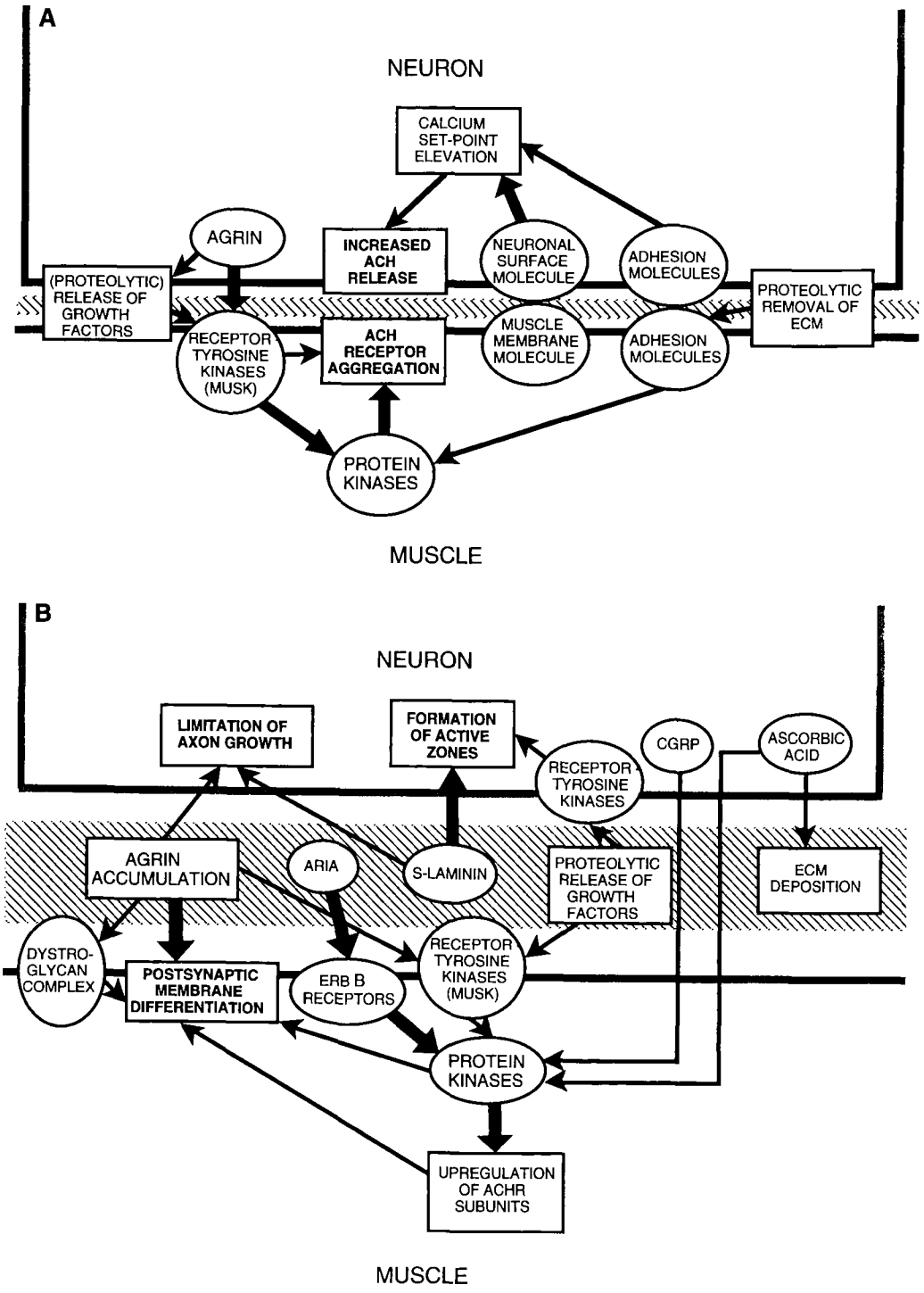

Fig. 1. Simplified representations of the earlier **(A)** and the later **(B)** phase of early inductive interactions resulting in NMJ formation. Inductive events or processes are represented by rectangles, and the critical events are in bold type. Molecules or types of molecules are represented by ellipses. ECM is represented by the hatchmarks between the nerve and muscle cell, and is a more prominent component in the later stage. For clarity, elements represented within the ECM in (B) were not connected with arrows, although they are likely to interact. The figure is intended to emphasize interactions and common pathways between signaling molecules and molecular processes that may be involved in the early stages of NMJ development. The interactions whose involvement are most strongly supported by experimental results are indicated by the thicker arrows.

Adhesive Interactions

Since many ligands and their receptors are bound to the cell surface or are integral membrane proteins, the strength of a cell–cell interaction may well be influenced by adhesion between the two cell surfaces, increasing the likelihood that receptor and ligand will come into contact. Thus, adhesive interactions could serve in a permissive role as well as in directly transducing intracellular events that lead to cell-surface differentiation. An example of the latter is found in the observation that the outgrowth-promoting activity of NCAM, L1, and N-cadherin is mediated through the activation of FGF receptors in neuritic growth cones when they grow over transfected fibroblasts expressing these adhesion molecules (Williams et al., 1994). Integrin-based adhesion between cells or cells and extracellular matrices also involves remodeling of the submembrane cytoskeleton and is associated with the accumulation of protein tyrosine kinases, such as a focal adhesion kinase and a member of the src family kinases (Romer et al., 1992; Parsons et al., 1994). In this respect, it is interesting that the formation of focal adhesion-like domains on the myotube surface where it contacts the culture substrate is coupled to the formation of immediately adjacent AChR-enriched domains containing the same cytoskeletal proteins found in the developing NMJ (Bloch and Pumplin, 1988; Pumplin, 1989; Bloch and Morrow, 1989; Bloch et al., 1991, 1994; Daniels et al., 1990).

Extracellular Matrix

The involvement of ECM molecules in the inductive process leading to NMJ formation might occur through diverse pathways. The simple fact that key molecules, such as acetylcholinesterase, agrin, and *S*-laminin, have domains that are structurally related to conventional ECM molecules is providing the basis for more detailed studies of the inductive role of the junctional basal lamina (Sanes, 1996). Growth factors (as well as agrin and ARIA) are deposited in the ECM and bind to its components, particularly HSPGs. It has been shown that proteolytic cleavage of the ECM can release growth factors in their active form (Saskela and Rifkin, 1990). Thus, a specific proteolytic cascade might result in the local activation of growth factors or similar molecules as well as in disturbance of existing ECM–receptor interactions (Anderson, 1986). Conversely, the enhancement of the deposition of ECM by ascorbate or (indirectly) by agrin could provide a repository for molecules whose continued activity would maintain the differentiated state. A recent report (Jones et al., 1996) that substrate-bound nerve or muscle isoforms of agrin can induce increased expression of the AChR ε subunit (as previously demonstrated for ARIA) is consistent with this idea.

Signal Transduction Pathways

It is noteworthy that the actions of different candidate ligands for both pre and postsynaptic induction involve convergent intracellular signaling pathways, notably the activation of protein tyrosine kinases. There is now considerable evidence that the induction of postsynaptic AChR aggregation by various stimuli, including positively charged beads, FGFb and HB-GAM-coated beads, applied electrical fields, and agrin all involve the phosphorylation of tyrosine residues on one or more membrane-associated proteins (Wallace, 1988; Peng et al., 1991, 1993, 1995; Baker et al., 1992; Baker and Peng, 1993; Wallace, 1994, 1995; Meier et al., 1995). This has been suggested by the accumulation of phosphotyrosine residues at or before the time of AChR aggregation, and by the inhibition of AChR aggregation by protein tyrosine kinase inhibitors. The identified substrates for tyrosine phosphorylation in cultured muscle cells include MuSK itself (Glass et al., 1996), AChR β, γ and δ-subunits, apparently the major tyrosine-phosphorylated species (Wallace et al., 1991; Qu and Huganir, 1994), and an 87-kDa protein homologous to the C-terminal portion of dystrophin (Wagner

et al., 1993). It is possible that AChRs and their associated proteins are phosphorylated directly by activation of receptor tyrosine kinases, such as growth factor receptors or MuSK. Alternatively, they may be phosphorylated indirectly through membrane-associated tyrosine kinases of the src family, such as fyn and fyk, which are found in a complex with AChR in the receptor-enriched membranes of *Torpedo* electroplax, can bind to and phosphorylate AChR subunits in vitro, and also are found in *Torpedo* skeletal muscle (Swope and Huganir, 1993, 1994). Interestingly, rapsyn alone expressed in *Xenopus* oocytes will form clusters that coincide with other proteins phosphorylated on tyrosine residues (Dai et al., 1996). Whereas there is a clear accumulation of phosphotyrosine at newly formed NMJs of chick, this seems to occur later in development in the rat. On the other hand, transient tyrosine phosphorylation of the AChR-β-subunit occurs prior to the accumulation of AChR in C2 myotubes exposed to agrin (Ferns et al., 1996). Thus, it is possible that tyrosine phosphorylation of the β-subunit represents a transient phase of the signal transduction process leading to AChR aggregation, but the phosphorylation of AChRs *per se* has not yet been demonstrated to be required for AChR aggregation in muscle cells. Signal transduction by different receptor tyrosine kinases is thought to be compartmentalized, in part through their interactions with specific src homology 2 (SH2) domain-containing proteins (Schlessinger and Ullrich, 1992), but this is not yet known to be true for MuSK vs the other receptor tyrosine kinases in myotubes. An interesting possibility that can be tested by use of the MuSK deletion mouse is that both physiological and nonphysiological stimuli for AChR aggregation operate through the activation of MuSK. Ultimately, a more detailed and direct analysis of the signal transduction pathways involved in the induction of AChR aggregation by different stimuli should clarify not only the role of protein phosphorylation, but also the pathways through which various signaling molecules may act.

Prospects for Future Research

Pathways and Mechanisms of the Identified Participants

Research on the signaling mechanisms utilized by the motoneuron and muscle cell during synaptogenesis has progressed from the initial discovery of a reciprocal inductive process to the identification of some of the molecules likely to be involved, and the beginnings of a description of the molecular interactions and signal transduction pathways leading to pre- and postsynaptic differentiation. Recent "knockout" experiments have clearly demonstrated the involvement of agrin, MuSK, and rapsyn in the induction of postsynaptic differentiation, and of *S*-laminin in the maturation of the presynaptic nerve terminal. Undoubtedly, it is important to study in detail the mechanisms by which these molecules act, and these lines of research are being pursued enthusiastically by a number of laboratories. Little is known about the spatial and temporal regulation of the expression of these proteins. In the case of agrin, for example, it has not been determined whether the protein is specifically targeted to axons, how it is secreted, or the means by which its gene transcription is regulated. It is equally important to obtain further evidence regarding the role of other molecules that have been putatively implicated in synaptic induction. ARIA, for example, has received much attention as a possible regulator of AChR gene expression in vivo, and the signal transduction pathway of its receptor is already the subject of two reports (cited above). However, the field awaits definitive experiments with blocking antibodies or genetic manipulation of ARIA expression to define ARIA's role better.

Presynaptic Differentiation

Most of the progress in the understanding of synaptic induction has been on the postsynaptic side, probably because of the relative ease of assaying AChR expression and aggregation.

Not enough is known about the supramolecular structure of the presynaptic terminal, much less the molecular signals that lead to its assembly. In particular, although many of the presynaptic proteins have been identified, the molecular composition of the dense active zone strucures is essentially unknown. This should be the subject of careful high-resolution immunocytochemical studies. Efforts should continue to isolate the signaling molecules generated by muscle cells that induce changes in presynaptic calcium levels, increases in ACh release, and the subsequent maturation of the presynaptic apparatus. The roles of molecules putatively involved in presynaptic maturation, S-laminin, agrin, and growth factors, such as HB-GAM, FGFb, and neurotrophins should be carefully evaluated.

Less-Studied and Novel Molecules

It would be shortsighted for researchers in the field to overlook molecules and mechanisms whose involvement in synaptic induction have been suggested by well-documented results of only one or two laboratories. These include proteases, heparin binding growth factors, adhesion molecules, and smaller molecules, such as ascorbic acid. Whether or not these molecules act as primary signals for synaptic differentiation, their interactions with other molecules at the developing synapse (discussed above) probably regulate the formation and stabilization of pre- and postsynaptic structures. Finally, investigators of vertebrate NMJ development might do well to take advantage of the information that is emerging from molecular genetic studies of NMJ formation in *Drosophila* (*see*, for example, Kopczynski et al., 1996). Although these and other invertebrate NMJs are glutaminergic rather than cholinergic, it is likely that there are molecular signals and mechanisms common to synaptic induction in vertebrates and invertebrates. Thus, as the proteins involved in *Drosophila* NMJ formation are identified, it will be most interesting to examine their vertebrate homologs. In the last several years, there have been rapid advances in the understanding of molecular interactions involved in the postsynaptic clustering of glutamate and glycine receptors in the mammalian CNS (Ehlers et al., 1996; Kuhse et al., 1995), but the signaling molecules that trigger this clustering remain unknown. It would not be surprising if clues to the identity of these molecules emerged from neurogenetic studies of *Drosophila* NMJ development.

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