SIGNALING AT THE NEUROMUSCULAR SYNAPSE

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ABSTRACT

The vertebrate neuromuscular synapse arises as a result of inductive interactions between muscle and nerve. Accumulation of acetylcholine receptors (AChR) in the postsynaptic membrane is a hallmark of postsynaptic specialization. Motor neuron induces this specialization through three independent pathways. The first pathway is mediated by agrin, a basal lamina protein secreted by nerve, which triggers redistribution of AChRs to synaptic region of the muscle fiber. In the second pathway, motor nerve releases a signal that stimulates transcription of AChR genes by the synaptic muscle nuclei. Finally, motor neuron-evoked electrical activity suppresses the transcription and synthesis of AChRs by all the myofiber nuclei. Studies described in this thesis provide strong evidence that neuregulin, a protein originally isolated as the ligand for erbB2 receptor, is the signal from motor neuron that activates synapse-specific transcription. We demonstrate by immnofluorescence that neuregulin is present in the neuromuscular synapse, and that, like the signal that activates synapse-specific transcription, neuregulin remains at the original synaptic sites following muscle and nerve degeneration. In addition, we show that neuregulin stimulates AChR δ subunit gene transcription in C2 myotubes. Furthermore, we show that potential receptors for neuregulin, erbB3 and erbB4, are concentrated in the synaptic sites. Therefore, neuregulin is a potential signal that mediates synapse-specific transcription, and erbB3 and erbB4 are likely the receptors in muscle to transduce the signal to the nucleus. To further understand how nerve-evoked activity regulates neuromuscular development, I used a subtractive-hybridization cloning strategy to isolate new muscle genes whose expression is regulated by motor neuron innervation. A few genes, including the recently discovered acute myeloid leukemia (AML1) gene, were found to increase in expression following denervation, whereas the level of expression of one gene, INN1, declines after denervation. AML1 expression rises with a time course similar to that of AChR δ subunit gene, and in cultured muscle cells, is absolutely confined to the nuclei, suggesting that it plays a role in regulating the expression of other muscle genes following denervation.

Thesis Supervisor: Dr. Steven J. Burden
Title: Senior Investigator (CBR, Harvard Medical School)

to my father and mother,

Weiqiu and Qiaozhi Zhu

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ABBREVIATIONS USED

ACh	acetylcholine
AChR	acetylcholine receptor
AChE	acetylcholine esterase
AML1	acute myeloid leukemia
ARIA	acetylcholine receptor inducing activity
bHLH	basic helix-loop-helix
CAT	chloramphenicol acetyltransferase
CBF β	core binding factor β
CGRP	calcitonin related-gene peptide
DEN	denervated
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
GGF	glial growth factor
HER3	human erbB3
hGH	human growth hormone
HRP	horseradish peroxidase
INN	innervated
MAP1a	microtubule associated-protein 1a
NDF	Neu differentiation factor
NGF	nerve growth factor
NMJ	neuromuscular junction
NSP	neuroendocrine-specific protein
PKC	protein kinase C
TGF-β	transforming growth factor β
TMR-α-BGT	tetramethylrhodamine α-bungarotoxin

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Chapter 1

General Introduction

INTRODUCTION

Synaptic transmission underlies nearly every aspect of the function of the nervous system. Understanding how the synapse is formed and maintained has become one of the central problems in neurobiology. Synapse development has been best studied in the vertebrate neuromuscular synapse, partly because of its relative simplicity and accessibility.

The neuromuscular synapse arises as a result of complex inductive interactions between muscle and motor neurons. The initial contact between a muscle fiber and a motor neuron triggers a series of events which culminates in the alteration of the structures of both cells to form a synapse. A mature neuromuscular synapse reflects many morphological specializations (Engel, 1986; Ogata, 1988). At the presynaptic side, the motor nerve terminals are filled with synaptic vesicles containing the transmitter, acetylcholine (ACh). Many synaptic vesicles are clustered against dense patches of the nerve terminal called active zones, sites at which the vesicles fuse with the membrane to release the transmitters (Torri-Tarelli et al., 1985). In addition to these synaptic vesicles, large dense vesicles which are thought to contain neuropeptides such as calcitonin gene-related peptide (CGRP) are also associated with the nerve terminal (Matteoli, et al., 1988, 1990). Numerous mitochondria are also present in the nerve terminal, which are thought to provide energy for transmitter synthesis and release.

The morphological specialization of the postsynaptic apparatus is characterized by the formation of shallow grooves or gutters of the muscle membrane at which nerve terminals terminate, and 1 μm deep junctional folds (Matthews-Bellinger and Salpeter, 1978). The junctional fold membrane is not homogeneous. The top region of the junctional folds appears thickened and has a cytoplasmic fuzzy surface (Birks et al., 1960). The membrane thickening is believed to be due to an extraordinary density of acetylcholine receptors in the top region of the junctional fold (Fertuck and Salpeter, 1974, 1976; Sealock et al., 1984). The nondense membrane at the lower part of the junctional folds has a 10-100 fold lower AChR density and is rich in voltage-gated sodium channels (Fertuck and Salpeter, 1976; Betz et al., 1984; Beam et al., 1985; Flucher and Daniels, 1989). In addition, myofiber nuclei tend to cluster beneath the nerve terminal. As will be discussed below, these nuclei are also distinct from other myofiber nuclei in that they transcribe a subset of genes at a higher rate.

The neuromuscular junction is also characterized by a high degree of biochemical specializations. Numerous molecules are found to be concentrated in the postsynaptic muscle fiber or synaptic basal lamina. A non-exhaustive list includes acetylcholine receptors (AChR, Gu and Hall, 1988), voltage-gated sodium channels (Flucher and

Daniels, 1989) and N-CAM (Covault and Sanes, 1986) in the postsynaptic membrane; 43 kd protein (Rapsin, Froehner et al., 1981) and a dystrophin-related-protein (utrophin, Ohlendieck et al., 1991) in juxtamembrane cytoplasm; and S-laminin (Hunter et al., 1989) and acetylcholinesterase (AChE, McMahan et al., 1978) in the basal lamina. Among them, the nicotinic acetylcholine receptors are the best characterized and have served as the model system for studying the mechanism of postsynaptic differentiation.

Acetylcholine receptors were the first ion channel to be purified and molecularly characterized. The discovery and development of α-bungarotoxin, which binds to AChRs with high affinity (Lee, 1979), made this possible. In addition, the electric organ of a marine ray, *Torpedo* , is densely innervated by cholinergic nerves and has provided a rich source of synaptic proteins. The AChRs were purified from the electric organ of *Torpedo* by affinity chromatography using immobilized α-bungarotoxin. The purified AChR is a pentamer consisting of four different subunits $\alpha_2\beta\gamma\delta$.

Based on the partial protein sequence derived from the purified receptor subunits (Raftery et al., 1980), the cDNAs encoding the Torpedo AChR receptor subunits were cloned (Noda et al., 1983). Deduced amino acid sequences indicated that the subunits are homologous and are probably derived from a common ancestral gene. To confirm that the cloned cDNAs actually encode a functional AChR, RNAs were transcribed from the cloned cDNAs and were injected into *Xenopus* oocytes to express the protein. These electro-physiological and biochemical analyses demonstrated that the injected oocytes express a channel that had the expected electrical physiological properties and bound α-bungarotoxin with high affinity (Mishina et al., 1984). Mammalian homologs were subsequently isolated and shown to be about 80% homologous to their *Torpedo* counterparts (Takai et al., 1985; Mishina et al., 1986).

Development of the neuromuscular synapse

Development of the neuromuscular synapse can be divided into several stages, including initial establishment of the synapse and subsequent maturation of the synapse. These topics will be discussed separately below:

Initial establishment of the synapse

Muscle differentiation and synapse formation occur concomitantly. In the developing rodent embryo, motor axons enter muscle masses at embryonic days 14 to 15, at which time the myoblasts are fusing to form multinucleated myotubes (Kelly and Zacks, 1969). As the motor neuron approaches the developing muscle fiber, its terminal loses its myelin sheath and elaborates many fine processes. In the developing muscle fiber, AChR

expression is initiated as a result of the myogenic differentiation program. Initially AChRs are distributed diffusely over the entire muscle surface, reaching a density of approximately several hundred per μm^2 (Bevan and Steinbach, 1977; Burden, 1977; Steinbach, 1981). By the time the first contacts between motor neurons and muscle fibers are made, synaptic potentials can already be detected in the myofiber membrane (Diamond and Miledi, 1962; Dennis et al., 1981; Chow and Poo, 1985). Since active zones in the presynaptic terminal have not fully developed at this stage, it is believed that the initial quantal release of ACh does not depend on fully differentiated active zones.

The initial nerve-muscle contact causes the localization of AChRs and AChEs to the primitive synapse. By E16, AChRs are concentrated in an aggregate beneath the nerve terminal at a density of ~2500 per μm^2 (Mathews-Bellinger and Salpeter, 1983) and deposits of AChE can also be detected around the nerve terminals (Rubin et al., 1979; Sanes and Chiu, 1983). Between E16 and birth, myofibers become multiply innervated by several axons that converge at the single synaptic site (Redfern, 1970). The density of AChRs in the synaptic region increases to 10,000 per μm^2, comparable to that of an adult synapse (Mathews-Bellinger and Salpeter, 1983). During the same period, the collagen-tailed form of AChE increases in its level and becomes more concentrated in the synaptic region (Steinbach, 1981; Koenig and Rieger, 1981).

Synapse maturation

The structure of the synapse continues to be refined postnatally and does not reach full maturation until almost 1 month after birth. AChRs becomes more stabilized. The half-life of rat embryonic and fetal AChRs is about 1 day (Berg and Hall, 1975; Steinbach et al., 1979; Reiness and Weinberg, 1981; Steinbach, 1981). Between E18 and birth, the half-life of AChRs in the synaptic region increases to approximately 10 days; while the half-life of non-synaptic AChRs remains at about 1 day (Michler and Sakmann, 1980; Reiness and Weinberg, 1981; Steinbach, 1981). Synaptic AChR clusters also become progressively more resistant to dispersal by denervation (Slater, 1982), low Ca^{2+}, or elevated KCl (Bloch and Steinbach, 1981; Braithwaite and Harris, 1979). Two weeks after birth, AChR expression in the non-synaptic region decreases to an almost undetectable level, a process believed to be mediated by nerve-evoked electrical activity. At this time, the density of junctional AChRs is about 1000-fold greater than that of non-synaptic AChRs, and the junctional AChRs have changed in gating characteristic, from low-conductance, long open-time to high-conductance, short open-time channel (Fischbach and Schuetze, 1980). This change in gating characteristic is accompanied by a switch of one of the receptor subunits, from $\alpha_2\beta\gamma\delta$ to $\alpha_2\beta\varepsilon\delta$ (Mishina et al., 1986; Gu and

Hall, 1988). In rodents, the junctional folds appear about one week after birth and become more prominent through the next few weeks (Bixby, 1981). At the same time that junctional folds develop, the number of terminal profiles per endplate declines, consistent with the elimination of multi-terminal innervation (see below).

Synapse elimination

During early neuromuscular development, each muscle fiber is initially innervated by multiple axons at one synaptic site; whereas at a mature synapse, all but one motor axon is eliminated. This reduction in the number of motor axons innervating a single muscle fiber during normal development is called synapse elimination.

Evidence suggests that muscle activity plays a role in synapse elimination. Paralysis of chick embryo muscles with curare results in not only multiple axonal innervation at end-plates but also the development of extra innervated end-plates scattered along the length of individual fibers (Srihari and Vrbova, 1978; Ding et al., 1983; Oppenheim and Chu-Wang, 1983). Concomitantly, the number of muscles innervated by a single motor axon increases by about 40% (Ding et al., 1983). Similar results are also obtained with rat muscle. Blockade of sciatic nerve action potentials with tetrodotoxin (Thompson et al., 1979) or paralysis of muscles by injection of botulinum toxin to block ACh release (Brown et al., 1982) inhibits the elimination of synapses in postnatal rat muscle. On the other hand, enhanced electrical activity increases synapse elimination. For example, artificial stimulation by implanted electrodes of neonatal rat soleus muscle has been shown to accelerate synapse elimination in soleus muscle (Thompson, 1983). In this case, the most effective stimulation was delivered by high-frequency trains of activity (Thompson, 1983), whereas low-frequency activity is less effective.

Despite the evidence for a role of electrical activity in synapse elimination, the exact underlying mechanism is still not known. Recent studies, however, have provided some interesting insights into this process. In one of these studies, elimination of multiply innervated synapse in reinnervated neuromuscular synapse was observed in real time (Rich and Lichtman, 1989a, 1989b). When motor axons regenerate following axotomy, many muscle fibers become reinnervated by more than one motor neuron; most of these extra contacts are subsequently withdrawn (McArdle, 1975; Gorio et al., 1983; Werle and Herrera, 1988), which mimics synapse elimination during normal development. In this study, the presynaptic nerve terminal and the postsynaptic AChRs were visualized with the mitochondrial dye 4-Di-2-ASP and rhodamine-conjugated α-bungarotoxin respectively. A surprising finding from these studies is that AChRs started to disappear from particular regions of the junction before any noticeable change occurred in their

associated nerve terminals. The fact that the disappearance of AChRs precedes synapse elimination suggests that AChRs might play a role in destablization of synapses that eventually leads to elimination.

In another study, a nerve-muscle co-culture from *Xenopus* embryos was used to demonstrate that a hebbian mechanism may underlie synapse elimination (Lo and Poo, 1991; Dan and Poo, 1992). According to this theory, a nerve terminal that depolarizes synchronously with its postsynaptic muscle cell would become stabilized, whereas a terminal that depolarizes asynchronously with the muscle cell would become destabilized. In culture, when a single muscle cell is innervated by two neurons, repeated stimulation of one neuron leads to a decrease in the strength of the synapse made by the other neuron. If both neurons are stimulated synchronously, minimal suppression is observed. However, if the stimulation is asynchronous, one or both synapses are suppressed. Quantal analysis indicates that suppression is due to a reduction in ACh release, rather than a decrease in the responsiveness by the muscle cell. This suggests that a short-lived retrograde messenger derived from muscle might pariticipate in weakening the release of neurotransmitters from the presynaptic terminal. Unfortunately, the nerve-muscle co-culture could not be maintained long enough to allow an examination of synapse elimination by morphological criteria.

Mechanism of accumulation of AChRs at the synapse

As mentioned earlier, AChRs are highly concentrated in the synaptic region of the muscle fiber. At a mature neuromuscular junction, which occupies less than 0.1% of the myofiber surface, the AChR density exceeds 10,000 per μm^2, whereas outside this region, the level drops sharply to no more than about 10 AChR per μm^2. Accumulation of AChRs at the synapse has been the object of intense study for the past few decades, and these studies point to three independent pathways that contribute to the localization. In the first pathway, agrin, a basal lamina protein synthesized by motor neuron, triggers a redistribution of pre-existing AChRs to the synaptic region. In the second pathway, motor neurons stimulate synaptic muscle nuclei to transcribe AChR subunit genes at a higher rate than the rest of the myofiber nuclei, via an unknown factor. Finally, motor nerve-evoked electrical activity suppresses transcription of AChR subunit genes by the all the muscle nuclei, with only synaptic nuclei maintaining a high transcription rate due to local stimulation.

Agrin-induced accumulation of AChRs

Early studies suggest that factors secreted by motor neuron can induce

redistribution of AChRs and promote formation of AChR clusters, since motor neurons co-cultured with myotubes induce the redistribution of pre-existing AChRs to the newly formed synaptic sites (Anderson and Cohen, 1977). Subsequent studies of regenerating muscle showed that a signal associated with the synaptic basal lamina can induce clustering of AChRs at synaptic sites (Burden et al., 1979; Bader, 1981; McMahan and Slater, 1984). In these studies, both the nerve and muscle were damaged to induce degeneration, which eliminates the pre-existing muscle fibers and the motor nerve terminals. The only structure that survives this degeneration process is the myofiber's basal lamina sheath. Weeks later, when the muscle satellite cells proliferate and fuse to form new myofibers within the original basal lamina sheath, AChRs re-accumulate at the original synaptic site, even when the nerve is prevented from re-innervating the muscle. Since the basal lamina sheath is the only structure that remains intact during the entire degeneration-regeneration period, this result implies that a signal for clustering AChRs is associated with the synaptic basal lamina.

A search for the AChR clustering activity in the extracellular matrix of *Torpedo* electric organ, a rich source of synaptic molecules, led to the identification of agrin (Godfrey et al., 1984; Nitkin et al., 1987). Like the inductive signal at the developing neuromuscular synapse, agrin triggers AChR clustering by post-translational mechanisms. Soluble agrin induces clustering of AChRs and many other synaptic proteins, including the 43 kd protein and AChE, on cultured myotubes (Godfrey et al., 1984; Wallace, 1989b). When locally applied, it induces AChR clusters at the site of application (Campanelli et al., 1991). Agrin is synthesized by motor neurons and is transported along motor axons to the nerve terminal, where it is released and becomes incorporated into the synaptic basal lamina (Magill-Solc and McMahan, 1988, 1990; Reist et al., 1987). These results strongly suggest that motor neuron-derived agrin is responsible for clustering AChRs in vivo. However, the role of nerve-derived agrin in AChR accumulation became uncertain when it was discovered that muscle also produces agrin (Fallon and Gelfman, 1989; Lieth et al., 1991). Definitive evidence that nerve-derived agrin is responsible for clustering AChRs at developing neuromuscular synapses comes from a chimeric nerve-muscle co-culture experiment (Reist et al., 1992). Antibodies specific for chick agrin blocks the formation of AChR clusters in chick nerve-rat muscle co-cultures, indicating that nerve-derived agrin is required for clustering AChRs. The same antibodies, however, failed to block the formation of AChR clusters in rat nerve-chick muscle chimeric culture, demonstrating that muscle-derived agrin is dispensable for AChR clustering.

Cloning of agrin cDNAs reveals some interesting structural domains within this protein. At present, agrin has been cloned from a variety of species, including *Torpedo*,

chick, mouse and rat (Rupp et al., 1991; Tsim et al., 1992; Rupp et al., 1992). It contains several epidermal growth factor (EGF)-like domains, two different laminin domains and multiple follistatin-like repeats. The EGF-like domains are contained within the carboxyl-terminal region, which has been shown to be sufficient to induce AChR clusters on cultured myotubes when applied as a soluble factor, suggesting that these domains might be directly involved in agrin-mediated signaling. The role of the laminin domains is not clear; perhaps they are responsible for association of agrin with the extracellular matrix.

Alternative splicing of agrin generates multiple isoforms that differ in their AChR clustering efficiency (Ruegg et al., 1992; Ferns et al., 1992; Ferns et al., 1993; Hoch et al., 1993). These isoforms differ primarily at three different splicing sites, termed X (3 or 12 amino acids), Y (4 amino acids), and Z (8 or 11 amino acids) respectively. The presence or absence of Y and Z splicing sites most profoundly affect the AChR clustering ability, whereas X has no effect. Agrin that lacks both Y and Z splicing sites has no clustering activity on chick myotubes, whereas inclusion of 8 amino acids at the Z site almost restores near full activity. Surprisingly, rat primary myotubes and C2 myotubes behave differently from chick myotubes in that they respond to agrin lacking Y and Z sites (Ferns et al., 1993). Proteoglycans seem to play a role in agrin-mediated AChR clustering, since a C2 cell line that is deficient in proteoglycan synthesis fails to respond to agrin isoforms that lack Y and Z splicing sites but responds normally to isoforms containing the Z splicing sites. The only cells known to express the isoform with all three splicing sites are neurons. Muscle and various other tissues express isoforms that lack any one or two of the Y and Z sites. The function of these isoforms is unknown.

The mechanism by which agrin clusters AChRs is not known. Several studies suggested a role for phosphorylation. Agrin stimulates tyrosine phosphorylation of the β subunit of AChR with a time course that parallels agrin-mediated clustering of AChRs (Wallace et al., 1991). Moreover, herbimycin, an inhibitor of tyrosine phosphorylation, blocks both agrin-induced AChR phosphorylation and AChR clustering (Qu et al., 1993). Since herbimycin inhibits tyrosine phosphorylation of a variety of substrates, this result does not allow a direct evaluation of the role of AChR tyrosine phosphorylation in agrin-induced clustering. In addition to tyrosine phosphorylation, there is evidence for serine phosphorylation in agrin-mediated signaling pathway, since activation of protein kinase C (PKC) by phorbol ester inhibits agrin-induced AChR clustering (Wallace, 1989a). It is not known whether the activities of PKC and other serine kinases are regulated by agrin.

Most recently, a putative receptor for agrin has been identified and shown to be dystroglycan (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), a membrane protein that co-purifies with dystrophin (Ervasti et al., 1991) and

utrophin (Matsumura et al., 1992), a dystrophin-related protein that is highly concentrated at nerve-muscle synapses (Ohlendieck et al., 1991; Phillips et al., 1993). Dystroglycan binds agrin with high affinity and is co-extensive with agrin-induced AChR clusters. However, there is controversy regarding whether dystroglycan is the functional agrin receptor. Conflicting results were reported regarding the effect of an anti-dystroglycan antibody, IIH6, which blocks agrin binding to dystroglycan, in blocking agrin-mediated AChR clustering (Gee et al., 1994; Sugiyama, et al., 1994). Furthermore, since one agrin molecule is capable of clustering a greater number of AChR molecules (Nitkin et al, 1987), signal amplification is thought to play a role in agrin-mediated clustering, yet dystroglycan lacks any known catalytic domain that is capable of such signal amplification. It is possible that agrin receptors, like receptors for FGF, NGF and TGF-β, consist of more than one subunit. By analogy with FGF, which binds to both proteoglycan and FGF receptor, it is possible that dystroglycan acts to concentrate agrin, whereas another unknown signal-transducing receptor serves the signal transduction function.

Rapsin, a 43 kd protein that is present at a 1:1 stoichiometry with AChRs at the synaptic sites, has been shown to be able to cluster AChRs in some cell types (Froehner, 1991). Rapsin forms spontaneous clusters when it is expressed in either *Xenopus* oocytes or fibroblasts. Moreover, if AChRs are co-expressed in these cells, Rapsin can induce AChR clusters, which co-localize with Rapsin clusters. Rapsin is a peripheral membrane protein that is in close association with AChRs. Selective extraction of this protein under alkaline condition reduces the stability of AChRs clusters, suggesting that it plays a role in stabilizing high density of AChRs. Agrin also induces formation of Rapsin clusters, which almost always co-localize with AChR clusters (Wallace, 1989b). The exact role of Rapsin in agrin-mediated AChR clustering has not been established. But given the ability of Rapsin to promote AChR cluster formation, it would not be surprising that agrin stimulates interaction between AChRs and Rapsins, perhaps via posttranslational modification of either molecule, such as phosphorylation.

Synapse-specific gene transcription

When the AChR α and δ subunit mRNA levels were measured in synapse-rich and synapse-free regions of the mouse diaphragm muscle, they were found to be enriched in the synapse-rich region (Merlie and Sanes, 1985). This early experiment inspired subsequent in situ hybridization studies examining the distribution of AChR subunit mRNAs in muscle fibers (Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). The general conclusion from these studies is that

AChR α, β, ε and δ subunit mRNAs are highly concentrated near synaptic nuclei and are nearly absent from the non-synaptic region. These findings immediately suggest that local synthesis also contributes to the high concentration of AChRs in the synapse. In theory, this local accumulation of AChR mRNAs could arise as a result of several mechanisms. For example, the AChR genes could be selectively transcribed by the synaptic nuclei; alternatively, AChRs could be transcribed at similar rates by all myofiber nuclei, but the transcripts could be selectively stabilized in the synaptic region. Transgenic studies indicate that transcriptional mechanisms are, at least in part, responsible for the concentration of AChR mRNAs at synaptic sites (Sanes et al., 1991; Simon et al., 1992). In one such study, 1.8 kb of AChR δ subunit upstream regulatory region was fused with a reporter gene, human growth hormone (hGH) and the gene fusion was introduced into mice (Simon et al., 1992). Muscle fibers do not normally express hGH, but they possess the machinery for processing hGH through the secretory pathway. The end result is that hGH is found transiently in the Golgi apparatus, which surrounds the myofiber nuclei and gives a characteristic perinuclear staining pattern. When muscle fibers from these transgenic mice were stained with antibodies to hGH, staining was virtually confined to the synaptic nuclei. As a control, when muscle fibers from transgenic mice carrying a gene fusion between the muscle creatine kinase promoter and hGH were analyzed, the staining was uniform throughout the muscle fiber. Therefore, local accumulation of hGH depends on AChR δ regulatory sequences. Likewise, when a 3.5 kb AChR ε subunit upstream sequence is fused to lacZ gene and is introduced into mice, the reporter gene product is again highly concentrated near synaptic nuclei (Sanes et al., 1991).

Muscle degeneration and regeneration experiments indicate that the signal that directs synapse-specific transcription is stably associated with the synaptic basal lamina (Goldman et al., 1991; Jo and Burden, 1992; Brenner et al., 1992). In one such study, the mouse sternomastoid muscle from the AChR δ-hGH transgenic mice was denervated by cutting the nerve innervating the muscle; at the same time, the synaptic region of the muscle was damaged by repeated freezing and thawing. This procedure results in degeneration of the myofibers, nerve terminal and other cells associated with the synapse, including Schwann cells and specialized fibroblast-like cells. The motor nerve was prevented from reinnervating the muscle by suturing the cut nerve to a nearby muscle, and the muscle was allowed to regenerate within the original basal lamina sheathes. hGH staining reappeared at the original synaptic sites in the regenerating muscle fibers, indicating that a stable signal that is associated with the synaptic basal lamina is responsible for synapse-specific transcription.

Although agrin has been shown to be associated with synaptic basal lamina and is responsible, at least in part, for clustering AChRs at the original synaptic sites following muscle degeneration and regeneration, it is most likely not the molecule that induces synapse-specific transcription. Agrin does not increase the synthesis of AChRs on cultured myotubes (Nitkin et al., 1987), and it does not stimulate AChR transcription in cultured muscle cells (Chu et al., 1995).

Several factors that stimulate AChR synthesis have been identified as candidates that might be responsible for synapse-specific transcription (Markelonis, et al., 1982; Knaack et al., 1986; New and Mudge, 1986; Jessell et al., 1979). Among these candidates, two molecules, calcitonin gene-related peptide (CGRP) and acetylcholine receptor inducing activity (ARIA), have received the most attention. CGRP increases AChR synthesis in cultured chick myotubes and is present in spinal motor neurons (New and Mudge, 1986; Fontaine et al., 1986). Its stimulatory effect, however, is small (50%) and is limited to α subunit (Fontaine et al., 1987). Moreover, despite its effect on AChR synthesis on chick myotubes, CGRP has no effect on the level of AChR mRNAs in mouse myotubes (Martinou, et al., 1991).

ARIA was originally purified from chicken brain on the basis of its ability to stimulate the rate of AChR incorporation in cultured chick myotubes (Usdin and Fischbach, 1986; Falls et al., 1990). It remains the most promising candidate since it mimics the endogenous factor in a number of aspects. First, ARIA increases the rate of AChR incorporation up to 10-fold and upregulates the mRNA level for AChR α subunit in cultured chick myotubes (Harris, et al., 1988, 1989). Moreover, ARIA elevates the mRNA level for AChR α, γ, δ (2-fold) and ε subunit (7-fold) in primary mouse myotubes by several-fold, with the most potent effect on ε subunit gene (Martinou et al., 1991). In rodents, developmental regulation of ε expression is distinct from the other subunits in that its appearance requires innervation and its mRNA is always confined to the synaptic nuclei (Brenner et al., 1990). Therefore, it has been suggested that the appearance of ε subunit is strictly dependent on a neurotrophic factor; this is consistent with ARIA's prominent effect on ε subunit in vitro.

ARIA cDNA was cloned from chick brain and was shown to be encoded by the same gene that encodes Neu differentiation factor (NDF), glial growth factor (GGF) and heregulin (Falls et al., 1993; Wen et al, 1992; Marchionni et al, 1993; Holmes et al., 1992). To avoid confusion over the terminology, a new term, neuregulin, was coined to replace the above nomenclature (Peles and Yarden, 1993). In situ hybridization and Northern blot analyses indicate that neuregulin is highly expressed in spinal motor neurons (Falls et al., 1993; Marchionni et al, 1993), consistent with the idea that it plays a

role in inducing the AChR synthesis in the synaptic region of muscle fibers. However, it is not known whether neuregulin is transported to the motor nerve terminal and whether it is incorporated into the synaptic basal lamina. As part of my thesis work, we used antibodies to demonstrate that neuregulins are present in the synaptic basal lamina, and that, like the signal in the basal lamina, neuregulin is capable of activating AChR δ subunit gene transcription. In addition, the same regulatory region that confers synapse-specific transcription of the AChR δ subunit gene in transgenic mice is sufficient to confer responsiveness to neuregulin. Moreover, we found that potential receptors for neuregulin, erbB3 and erbB4, are concentrated at the synaptic sites. These results suggest strongly that neuregulin is the endogenous molecule that stimulates synapse-specific transcription of AChR genes.

Electrical activity-dependent regulation of AChR transcription

Unlike the receptors in the synaptic region, AChR expression in the extrasynaptic region of the muscle fiber is profoundly affected by the electrical activity. Denervation causes a substantial increase in the number of AChRs in the nonsynaptic region, which accounts for so called denervation-induced hypersensitivity. Direct stimulation of denervated muscle prevented the appearance of non-synaptic AChRs, indicating that a loss of electrical activity is responsible for the appearance of non-synaptic AChRs (Lomo and Rosenthal, 1972). The appearance of extrasynaptic AChRs is a result of new protein synthesis, rather than redistribution of synaptic AChRs. In fact, synaptic receptor density remains unchanged for several weeks following denervation (Frank et al., 1976).

Results from many experiments implicate transcription mechanisms in electrical activity-dependent regulation of AChR subunit genes. The abundance of AChR subunit mRNAs increases upon denervation (Klarsfeld and Changeux, 1985; Goldman et al., 1988). Nuclear run-on experiments and transgenic mice studies provide unequivocal evidence for this idea (Tsay and Schimidt, 1989; Merlie and Kornhauser, 1989; Simon et al., 1992). In one study with transgenic mice, the leg muscle of mice harboring a gene fusion between 1.8 kb of AChR δ subunit regulatory sequence and a reporter gene hGH was denervated, and the hGH mRNA levels were measured before and after denervation. Like the endogenous AChR δ subunit, the level of hGH mRNA increases substantially following denervation, indicating that the 1.8 kb regulatory sequence is sufficient to confer electrical-activity dependent regulation.

Subsequent studies with both cultured myotubes and transgenic mice have shown that 181 bp regulatory sequence from AChR δ subunit is sufficient to confer electrical activity-dependent regulation (Simon et al., 1992; Dutton et al., 1993). Further study

demonstrated the importance of a proximal E-box for the electrical activity-dependent response (Tang et al., 1994). In transgenic mice, a mutation in the proximal E-box significantly attenuates the ability of the reporter gene to respond to denervation. Nevertheless, it is known that E-boxes are not sufficient to confer activity-dependent regulation, since muscle genes such as the creatine kinase and myosin light genes contain E-boxes in their upstream regulatory region, yet they are not regulated by denervation (Lassar et al., 1989; Donoghue, et al., 1988). It is believed that additional cis-elements are required to confer the electrical activity-dependent regulation..

In order to identify additional transcription factors that might mediate electrical activity-dependent regulation and muscle genes which mediate biological processes associated with innervation, I used a subtraction cloning strategy to identify new muscle genes whose expression is regulated by innervation. One of the genes identified in this screen, the acute myeloid leukemia (AML1) gene, is markedly induced by denervation. Because AML1 is a transcription factor, it may have a role in regulating expression of other muscle genes following denervation.

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Chapter 2

Subtraction Cloning of Muscle Genes Regulated by Motor Nerve Innervation.

SUMMARY

Motor nerve-induced muscle electrical activity has profound effects on synapse formation and muscle physiology. Not only does it help restrict AChRs to the synaptic region by suppressing their expression in the extrasynaptic region, but it also plays a key role in processes such as motoneuron death, polyneuronal synapse elimination, and the appearance of secondary muscle fibers. Muscle activity is known to repress the expression of a dozen or so genes, although the mechanism by which it acts is still unknown. To understand how activity regulates neuromuscular development, I used a subtractive-hybridization cloning technique to identify and isolate new muscle genes whose expression is regulated by activity. Innervated and denervated muscle cDNAs were prepared and biotinylated to be used in a PCR-based subtraction protocol. Multiple rounds of subtractive hybridization were carried out to remove common cDNAs. The subtracted cDNAs were cloned into a plasmid vector to construct subtracted cDNA libraries. After 2 rounds of screening, I identified several known genes that are known to be regulated by denervation, and at the same time, I cloned six additional genes whose expression was not previously known to be regulated by electrical activity (table 2). Three of these genes, AML1, filamin and microtuble-associated protein 1a (MAP1a), are previously known genes; while the other three, DEN1, DEN2 and INN1, encode novel proteins.

INTRODUCTION

Nerve-induced muscle activity affects many aspects of neuromuscular development, including motor neuron death, synapse formation, muscle development and polyneuronal synapse elimination. Its importance in the development of the neuromuscular synapse was realized in early denervation and drug experiments. For example, muscle activity is essential for the formation of junctional folds, since treatment of immature neuromuscular junction (NMJ) with α-bungarotoxin (α-BGT) can prevent junctional fold development (Duxson, 1982), whereas direct electrical stimulation of the muscle can counteract this effect (Brenner et al., 1983; Lomo et al., 1984). Moreover, muscle activity is implicated in the differentiation of other components of the NMJ, such as AChE. Blocking the electrical activity eliminates the appearance of AChE (De La Porte et al., 1984).

Many experiments demonstrate the importance of muscle activity in the process of motoneuron death. Several different synaptic blocking agents, including ones that block presynaptically (e.g. hemicholinium-3) and postsynaptically (e.g. curare) have been applied to chick embryos during the period of motoneuron death. Results have

consistently shown that such blockade can virtually abolish motoneuron death (Laing and Presie, 1978; Pittman and Oppenheim, 1979; Creazzo and Sohal, 1979). In addition, if chick embryo muscle is electrically stimulated, both the rate and amount of motoneuron death are enhanced (Oppenheim and Nunez, 1982). Finally, a mutant mouse (muscular dysgenesis), characterized by virtually total skeletal muscle paralysis in utero, also shows little or no motoneuron death (Oppenheim et al., 1984).

There is evidence that muscle development depends partly on motoneuron innervation. The first muscle fibers that appear, called primary muscle fibers, do not depend on the presence of motor neurons for their initial appearance (Kelly and Zacks, 1969). Later, additional myofibers are formed in close physical association with the primary myofibers. The late myofibers are called secondary fibers and they form the majority of fibers in most adult muscles. Secondary fibers depend on nerve-evoked activity for their initial appearance. If nerve activity is chronically blocked, the production of secondary muscle fibers is sharply reduced (Harris, 1981; Oppenheim and Chu-Wang, 1983; McClennan, 1983; Harris and McCaig, 1984).

The emergence of slow muscle fibers, unlike fast fibers, requires normal innervation by slow motor neurons. Denervation of the predominately slow soleus muscle, for example, arrests production of adult slow myosin, and therefore leads to the appearance of fast muscle by default (Gambke, et al., 1983). It appears that muscle activity, rather than nerve-derived trophic factors, is responsible for appearance of slow muscle fibers, since paralysis of chick embryos with curare produces effects similar to denervation (Gauthier et al., 1984).

During the early phase of neuromuscular synapse formation, each muscle fiber is innervated by multiple motor axons at the same end-plate. Later, all but one of the axons are removed; this process is called synapse elimination. The end result of synapse elimination is that in the adult, each muscle end-plate is innervated by only one motoneuron. In mammals, roughly one third to three-fourths of the synapses initially formed are subsequently eliminated.

Like motoneuron death, synapse elimination is also influenced by muscle activity. For example, paralysis of chick embryo muscles with curare results in not only multiple axonal innervation at end-plates but also the development of additional end-plates scattered along the length of individual fibers (Srihari and Vrbova, 1978; Ding et al., 1983; Oppenheim and Chu-Wang, 1983). Concomitantly, the size of motor unit, measured by the number of muscles innervated by a single motor axon, increases by about 40% (Ding et al., 1983). Similar results are also obtained with rat muscle. Blockade of sciatic nerve action potentials with tetrodotoxin (Thompson et al., 1979), or paralysis of muscles by

injection of botulinum toxin to block ACh release (Brown et al., 1982), inhibits the elimination of synapses in postnatal rat muscle. On the other hand, enhanced electrical activity increases synapse elimination. For example, stimulation of neonatal rat soleus muscle by implanted electrodes has been shown to accelerate synapse elimination in soleus muscle (Thompson, 1983). In this case, the most effective stimulation was delivered by high-frequency trains of activity (Thompson, 1983), whereas low-frequency activity is less effective.

Another interesting phenomena that is associated with the loss of activity in muscle is motor axon sprouting. It is known that when a muscle fiber is denervated, it causes the neighboring motor axons to sprout (Brown et al., 1981; Brinnell and Herrera, 1981; Brown, 1984; Werning and Herrera, 1986). Once again, muscle activity seems to be the key element since direct stimulation of denervated muscle can reverse the major part of the sprouting response. There is evidence that denervation-induced nerve sprouting is mediated by diffusible factors secreted by inactive muscle (Slack and Pockett, 1981; Pockett and Slack, 1982; Torigoe, 1985; Kuffler, 1989). Although many candidate molecules, including IGF2 and CNTF, have been proposed to mediate this process (Caroni and Brandes, 1990; Gurney, et al., 1992), the potential role of these molecules in sprouting has not been definitively established.

Nerve-evoked muscle activity also contribute, although indirectly, to the localization of AChRs to the synaptic region of the muscle fiber. In adult skeletal muscle, the density of AChRs in the extra-junctional region is barely detectable and is estimated to be 1000-fold less than that at the junctional region (Fertuck and Salpeter, 1976). The appearance of extrasynaptic AChRs depends on the activity of the muscle. Denervation or paralysis lead to a dramatic increase in the number of extrajunctional AChRs, owing to new AChR synthesis (Brockes and Hall, 1975; Merlie et al., 1984). There is good evidence that muscle activity represses AChR synthesis at the level of transcription (Tsay and Schmidt, 1989; Merlie and Kornhauser, 1989; Chahine et al., 1992; Simon et al., 1992).

It is still not clear how electrical activity regulates AChR gene transcription. Recent studies demonstrate that an E-box in the AChR δ subunit control region is critical for activity-dependent gene regulation. A mutation in a single E-box close to the transcription start site in a gene fusion between AChR δ regulatory region and hGH dramatically reduces the response of hGH expression to denervation (Tang et al., 1994; Bessereau, 1994). This result suggests that myogenic factors participate in the upregulation of AChR δ subunit transcription following denervation. Consistent with this notion, mRNA levels for the myogenic factors are found to increase following denervation

(Eftimie et al., 1991; Witzemann and Sakmann, 1991; Piette et al., 1992). However, it is known that E-boxes are not sufficient to confer electrical activity-dependent transcription, because many genes expressed in skeletal muscle, such as muscle creatine kinase gene, whose expression is not affected by denervation, contain E-boxes in their upstream regulatory region. It is thought that additional cis-elements are necessary to mediate electrical activity-dependent regulation.

To identify muscle gene products that are associated with the biological changes following denervation, I undertook a subtractive-hybridization cloning approach to isolate muscle genes that are differentially expressed in innervated and denervated muscle. This chapter describes the isolation and characterization of several cDNAs identified by the screen, including DEN1, DEN2, whose abundance increases following denervation, and INN1, which is expressed at higher levels in innervated muscle than in denervated muscle.

MATERIALS AND METHODS

Preparation of RNA and cDNA

The lower leg muscles of adult Sprague-Dawley rats were surgically denervated by removing approximately 2 mm of the sciatic nerve. Total RNAs from denervated (5 days) or innervated lower leg muscle were isolated as described (Chomczynski and Sacchi, 1987). Poly $(A)^+$ RNA was purified with oligo(dT) cellulose (Aviv and Leder, 1972). Oligo$(dT)_{12\text{-}18}$ (Pharmacia) was used to prime the first strand cDNA synthesis from 2 ug of Poly $(A)^+$ RNA. The second strand was synthesized as described (Gubler and Hoffman, 1983). The average size of the cDNA was about 2 kilobases.

Muscle cDNA library construction

Innervated and denervated muscle cDNA libraries were constructed to ensure the isolation of full-length sequences using the isolated cDNA fragments as probes. Muscle mRNA was isolated as described above. First strand of cDNA was primed with an oligo(dT) primer containing a SalI site at the 5' end. The second strand was synthesized as described (Gubler and Hoffman, 1983). The cDNAs were blunt-ended with T4 polymerase and EcoR1 adaptors were ligated to the ends. The cDNAs were size selected and digested with SalI and cloned between the SalI, EcoRI sites of PBluescript II SK+. The average size of the inserts was approximately 1.5 kb.

Subtractive-hybridization-cloning

Innervated and denervated muscle cDNAs were digested with AluI and RsaI separately and pooled to produce short fragments appropriate for PCR amplification. Linkers bearing an EcoRI site were added to the blunt-ended cDNAs, and cDNAs in the range of 150 bp to 1 kb were gel-purified and pooled. PCR amplification, photo-biotinylation of driver DNA, hybridization and removal of biotinylated driver DNA were done as described (Wang and Brown, 1992), except that only 2 μg of target DNA and 40 μg of driver DNA were included in the hybridization reactions. Four rounds each of long and short hybridizations were carried-out and the subtraction efficiency was evaluated by probing subtracted innervated and denervated cDNA with denervated cDNA probe (cross-hybridization test). Denervated subtracted cDNA hybridized 40x stronger than the innervated cDNA, indicating that there is minimal shared sequences between the two subtracted libraries. The subtracted DNAs were digested with EcoRI and cloned into the EcoR1 site of pBluescript IISK+ to generate the subtracted libraries. The libraries were screened with random-primed ^{32}P-labelled subtracted probe, and positive colonies were then subjected to further analysis. DNA was sequenced with Sequenase, according to the manufacturer's (USB) instructions.

RNase protection assay

RNase protection assays were performed as described previously (Simon et al., 1992). Briefly, radiolabelled antisense RNA was produced by in vitro transcription and was hybridized with total RNA in hybridization buffer overnight at 42°C. Non-hybridized RNA was digested with RNase (A and T1) for 1 hour at 30°C. Following protease K digestion and phenol extraction, the protected RNA was precipitated with ethanol and fractionated in 5% denaturing polyacrylamide gels. RNase protection assays were quantitated with a PhosphorImager.

Northern blot analysis

mRNA was purified by oligo-dT cellulose chromatography (Aviv and Leder, 1972), electrophoresed in a 1% formaldehyde agarose gel and transferred to a GeneScreen (Du Pont) membrane. DNA probes were labelled by random-hexamer-priming. The membranes were hybridized overnight in 5XSSPE, 5X Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA at 68°C. The filter was washed in 0.1% SDS, 0.1X SSPE for 1 hour at 68°C and exposed for 2 days with an intensifying screen.

RESULTS

Development of a new subtraction strategy

The conventional subtraction scheme involves hybridization of cDNA from one pool with mRNA from another pool and subsequent removal of double-stranded cDNA-mRNA hybrids with hydroxyapatite. Although this technique has been used successfully to isolate differentially expressed mRNAs, the procedure is laborious and effective mostly for isolating abundant mRNAs. Two improvements have been made to circumvent the disadvantages associated with the classic technique. In one, the mRNA (termed driver) is biotinylated so that it can be easily removed by Streptavidin following hybridization, obviating the need for a cumbersome hydroxyapatite column (Sive and St. John, 1988). The other technique takes advantage of the ability of helper phage to make single-stranded DNA from plasmid. With this technique, cDNAs from two sources are cloned into the same vector in opposite direction to make single-stranded cDNA in opposite orientation. The single-stranded DNA from one source is biotinylated and used as driver to subtract the common sequences.

One technical problem associated with the later technique is the low efficiency of biotinylation of single-stranded DNA. I sought an alternative method to overcome this problem. This modification involves the use of in vitro transcribed biotinylated RNA instead of single-stranded DNA as driver. The RNA can be easily biotinylated by a 2-step reaction. Initially, an ATP analog, N6-aminohexyl-ATP (AH-ATP) is incorporated into RNA by RNA polymerase; the aminohexyl group is then converted into biotin by CAB-NHS ester. In my preliminary experiments, I found that transcription with 50% ATP and 50% AH-ATP produced a 20-30% incorporation of AH-ATP while the yield of RNA was only reduced 2-fold compared to that obtained with normal ATP. Subsequent reaction with CAB-NHS yielded over 50% biotinylation of the AH-AMP in the RNA, as determined by nearest neighbor analysis (Folsom et al., 1989). Streptavidin was added to this biotinylated RNA, and after two rounds of phenol-chloroform extraction, over 99% of the RNA was removed from the aqueous phase.

Using the purified cDNA and aminohexylated RNA driver, I did a pilot experiment to test the feasibility of the entire subtraction scheme. 1 ng of target DNA and 3 ng of aminohexylated driver RNA (about 10 fold molar excess) were used. Hybridization was carried-out in buffer containing 40% formamide at 52° C for 24 hours. I set up four hybridizations:

(1) Actin single-stranded DNA (antisense) from the synapse-specific library

(2) Actin single-stranded DNA (antisense) from the synapse-specific library with aminohexylated actin RNA (sense) transcribed from actin cDNA from the synapse-free library

(3) Single-stranded DNA (antisense) from a random clone in the synapse-specific library

(4) Single-stranded DNA (antisense) from a random clone in the synapse-specific library with aminohexylated actin RNA (sense) transcribed from actin cDNA from the synapse-free library

Because biotinylation of RNA decreases the rate of hybridization, in my experiments the aminohexylated RNA was derivatized with biotin only after hybridization. The standard procedures were then followed to remove the driver RNA and hybridized DNA. The remaining DNA were then converted to double-stranded cDNA and electroporated into E. Coli. The results showed that the actin driver RNA hybridized and removed most (98.5%) of the actin single-stranded DNA, resulting in over 60-fold reduction in the transformation efficiency of the actin DNA. The random clone single-stranded DNA, by contrast, showed little or no reduction in transformation efficiency (< 5%). Therefore, this subtraction technique is both efficient and specific.

Unfortunately, this technique failed when it was used in a subtraction between innervated and denervated muscle cDNAs to enrich for denervated muscle-specific mRNAs. Specifically, I found that the solubility of amino-hexylated RNA was much lower than normal RNA, and at approximately 1 ug/ul, the RNA gradually precipitated. Since the calculated half-time for hybridization of sequences of average abundance was about 1 day, the subtractive hybridization usually requires 4 to 5 days to be near complete. The biotinylated RNA was not able to remain in solution for this length of time. Therefore, I decided to explore other subtraction approaches.

Use of gene expression screen to identify genes preferentially expressed in either innervated or denervated muscle.

I used a PCR-mediated subtractive-hybridization and cloning method called gene expression screen (Wang and Brown, 1991) to identify genes which are expressed in skeletal muscle and which are regulated by innervation. One of the distinctive features of this technique is that it employs multiple rounds of hybridizations and therefore is very effective in removing common rare sequences. I prepared cDNAs from both innervated and denervated muscle, and used them in the subtractive hybridization (see Materials and Methods). Following 4 rounds of short and long hybridization (Table 1), I measured the efficiency of subtraction by probing subtracted innervated and denervated cDNA spotted on nitrocellulose filter with denervated cDNA (the cross hybridization test). The subtracted denervated cDNA hybridized 40-fold better than the innervated cDNA, indicating that there were few common cDNAs between the two subtracted cDNAs.

The subtracted innervated and denervated cDNAs were amplified by PCR. One aliquot of the amplified cDNA was cloned into pBLUESRIPT IISK+ to construct

subtracted cDNA libraries. Another aliquot was radiolabelled by random priming to screen the subtracted libraries to identify the most abundant clones in the subtracted libraries.

I initially screened twenty colonies from the denervated subtracted library; thirteen colonies contained cDNA fragments encoding αB crystallin, and the other seven colonies corresponded to cDNA fragments for MAP1a, filamin or AML1. To identify additional cDNAs contained in the library, I prepared a secondary denervated subtracted library by removing crystallin sequences from the original library with biotinylated crystallin cDNA fragments, and I screened nineteen colonies from this secondary subtracted denervated library. Eighteen colonies contained cDNAs encoding mRNAs that are expressed at higher levels in denervated than innervated skeletal muscle; these include cDNA fragments encoding AChR α and δ subunits, N-CAM, filamin, DEN1 and DEN2.

I screened the innervated subtracted cDNA library once with subtracted innervated cDNA probe and purified DNA fragments from 20 colonies that I screened. Subsequent RNase protection assays indicated that of the two clones that I analyzed, the abundance of one clone is indeed regulated by denervation. The clone was named as INN1. The abundance of the other clone is comparable in innervated and denervated muscle and is therefore, not regulated by innervation.

Characterization of the isolated cDNAs

To confirm that the isolated cDNA fragments correspond to mRNAs whose level of expression is altered by denervation, I performed a series of RNase protection assays to measure the abundance of isolated fragments in innervated and denervated muscle. The result is tabulated in table 1. In summary, all but one fragment (which encodes a ribosomal RNA) indeed encode mRNAs that are regulated by innervation, although they vary in the magnitude of response to denervation. A discernible trend is that mRNAs which are either more abundant (such as αB crystallin) or more responsive (such as *AML1*) to denervation are found more frequently in the subtracted cDNA library.

Isolation of full length cDNAs encoding DEN1, DEN2 and INN1

Using the short cDNA inserts prepared from DEN1, DEN2 and INN1 clones, Jim Yeadon, a graduate student in the lab, screened rat denervated and innervated muscle full-length cDNA plasmid libraries respectively, to obtain full-length sequence. Overlapping cDNA inserts were purified and sequenced. So far, we have obtained the entire coding sequence for INN1 (Fig. 1). We have isolated a full-length clone for DEN2 and have obtained near complete sequence. We have not yet obtained a full-length clone for DEN1,

and the obtained cDNA fragment was partially sequenced. Northern analysis of DEN1 indicated a single transcript of approximately 8 kb in denervated muscle (Fig. 2). The partial clone of DEN1 we isolated is approximately 3.4 kb.

To confirm that the longer cDNA clones isolated represent the same mRNAs encoded by the short cDNA fragments originally isolated from the subtracted library, we searched for the sequence of short cDNA fragments in the longer cDNA clones. In all three cases, we found the longer clones contain the short cDNA sequence. Therefore, DEN, DEN2 and INN1 represent mRNAs whose expression is regulated by innervation.

A search of the sequence database indicated that DEN1, DEN2 and INN1 are not identical to known DNA sequences in the database, and therefore, they encode novel cDNAs. DEN1 contains domains that are highly homologous to titin, a component of sarcomere. INN1 shares 65% identity in amino acid sequence with neuroendocrine-specific (NSP) gene, which is primarily expressed in neuronal and endocrinal cells. Based on the deduced amino acid sequence, INN1 contains two hydrophobic regions, suggesting that INN1 encodes a transmembrane protein.

Tissue distribution of INN1

To determine the tissue distribution of INN1, total RNA was isolated from a variety of tissues, and the abundance of INN1 in various tissues was measured by RNase protection assay. As shown in figure 3, INN1 expression is not restricted to innervated muscle, but rather, it is expressed in nearly every tissue examined (except liver, which has an abnormally low level of GAPDH signal as well). This expression pattern is in sharp contrast to NSP, whose expression is nearly restricted to the nervous system. This result suggests that INN1 and NSP might serve different functions in tissues, despite their resemblance in amino acid sequence.

Time course of DEN1 and DEN2 expression in response to denervation

To gain clues about the potential function of DEN1 and DEN2, the time course of their expression following denervation was determined. Total RNA was extracted from lower leg muscles 1, 2 or 5 days after denervation. The abundance of DEN1 and DEN2 mRNA was measured by RNase protection. Figure 4 shows that the level DEN1 and DEN2 expression does not change one day after denervation and increases modestly 2 days after denervation. Because a potential target gene, AChR δ subunit gene, increases in expression 1 day after denervation, the late onset in the rise of DEN1 and DEN2 expression suggests that they are most likely not involved in regulating the expression of other muscle genes after denervation.

DISCUSSION

Subtractive hybridization is a powerful technique to identify genes whose expression is altered by various physiological stimuli. It has been used successfully to isolate T-cell receptors (Hedrick et al., 1984), myogenic factors (Davis et al., 1987), and molecular markers for various developmental stages (Sargent and Dawid, 1983).

Several different subtractive hybridization schemes have been devised, each has its own advantages and shortcomings. Subtraction cloning using single-stranded cDNA as target and biotinylated mRNA as driver has the advantage of obtaining the full-length subtracted cDNA in the initial screen and thus desirable if a subsequent expression cloning scheme is to be used to select clones of particular interest. However, it is difficult to obtain a large amount of mRNA to serve as driver and therefore the concentration of the driver usually does not allow for near-complete hybridization; the subtraction efficiency is often unimpressive and therefore primarily suited for identifying abundant mRNAs. In comparison, the differential display method, which employs PCR amplification with an oligo dT primer and another 10-mer primer that anneals to different sites in different cDNAs, is fast and easy to perform. However, this method suffers the disadvantage of a high occurrence of false positives and an inability to obtain low-abundance mRNAs. In addition, since one set of primers anneal to the poly A tail of mRNA, the fragments amplified and isolated almost always correspond to the 3' untranslated sequence of an mRNA, making protein homology search pointless without obtaining further 5' sequence by a subsequent library screen.

The PCR based subtraction scheme, gene expression screen, offers the following advantages:

1) near exhaustive search for all mRNAs differentially expressed in both mRNA sources.
2) high incidence of obtaining coding sequence in the original screen to facilitate protein homology search.
3) high efficiency in removing common rare sequences and identifying low-abundance differentially expressed mRNAs.
4) high occurrence of true positives in the subtracted library screen to minimize screening efforts.

However, since the method employs multiple rounds of subtraction and amplification, it is relatively time-consuming and tedious. Furthermore, the isolated cDNAs are almost always short fragments and therefore require further library screens to obtain full-length sequence. Taken together, the vast advantages of this technique overwhelm the few disadvantages, making it a highly desirable technique for subtraction cloning.

I have screened the Den-Inn subtracted library twice and the Inn-Den library once to identify clones whose expression is regulated by innervation. So far, I have only obtained about one quarter of the genes whose expression is known to increase following denervation. This implies that my screening has not been exhaustive. It would be worthwhile to screen the library further by first removing the identified clones by subtraction.

It is interesting that the level of expression of filamin and MAP1a, both of which encode cytoskeletal proteins, increases following denervation. The synaptic region of muscle fiber is also characterized by accumulation of cytoskeletal proteins such as filamin, vinculin, α-actinin and ankyrin (Bloch and Hall, 1983; Marazzi et al., 1989; Cartaud et al., 1989). It has been proposed these proteins are involved in constructing a molecular scaffold to tether other localized proteins such as AChRs to the synaptic region. It is likely that nerve-induced activity contributes to the localization of some of the cytoskeletal proteins by repressing their synthesis in the extrasynaptic region. It shall be interesting to determine whether filamin, which has been shown to be concentrated at synaptic sites by immunohistochemistry, is also locally transcribed by the synaptic muscle nuclei. As a first step, it would be interesting to determine whether filamin mRNA is concentrated at synaptic sites using in situ hybridization.

One of the objectives for subtraction cloning is to identify genes that are involved in regulating the muscle's transcriptional response to denervation. The pathway that couples membrane excitation to nuclear transcription is not known. Nevertheless, recent studies strongly suggest the involvement of calcium and protein kinase C (PKC) in the pathway. It has been shown that the calcium channel blocker verapamil increases the level of AChR α subunit mRNA and AChR protein in cultured chick primary myotubes (Klarsfeld et al, 1989), whereas blocking the activity of PKC by prolonged treatment with TPA elevates the mRNA level for AChR α subunit gene (Klarsfeld et al, 1989). Moreover, electrical stimulation of denervated muscle increases the activity of nuclear PKC substantially and rapidly (Huang et al., 1992).

Recent studies with transgenic mice also implicate an E-box in the AChR δ subunit control region in activity-dependent gene regulation. A mutation in a single E-box close to the transcription start site in a gene fusion between AChR δ regulatory region and hGH dramatically reduces the response of hGH expression to denervation (Tang et al., 1994; Bessereau, 1994). This result suggests that myogenic factors are responsible for the upregulation of AChR δ subunit transcription following denervation. Consistent with this notion, mRNA levels for the myogenic factors are found to increase following denervation (Eftimie et al., 1991; Witzemann and Sakmann, 1991; Piette et al., 1992; Neville et al.,

1992). In addition, it has been suggested that PKC phosphorylate myogenin in electrically active cells and therefore abolishes the ability of myogenin to transactivate target genes.

Although myogenic factors might be critical for activity dependent gene expression, it is known that an E-box is not sufficient to confer electrical activity-dependent transcription, because many muscle specific genes which do not respond to denervation contain E-boxes in their upstream regulatory region. It is thought that additional cis-elements are necessary to confer the activity-dependent regulation. It follows that transcription factors other than myogenic factors might be involved in the activity-dependent regulation. If the level of expression of these putative transcription factors, like myogenin, changes as a result of denervation, the rise in the expression level would be expected to precede the increase in expression of their effector genes. Indeed, the level of myogenin mRNA increases with a faster time course than the AChR subunit genes. In this respect, subtraction of mRNAs between innervated muscle and muscle denervated for a shorter period time (e.g. 1 day rather than 5 days) might be more effective in identifying such genes.

Our sequence analysis indicated that INN1 is highly homologous (65%) to NSP. Yet INN1 and NSP have different expression patterns. In both rats and human, NSP expression is largely restricted to the nervous system. In contrast, INN1 expression is readily detectable in a variety of tissues examined. Unfortunately, our database sequence analysis did not reveal any known domains in INN1. At present, we do not know the subcellular localization of INN1, although the existence of two stretches of hydrophobic amino acids suggests that it is a transmembrane protein.

It is intriguing that the expression of INN1, unlike most other muscle genes, decreases following denervation. So far, only a few other genes, including GLUT-4, have been shown to behave in the same manner (Castello et al., 1993). Interestingly, an antigen recognized by a panel of monoclonal antibodies and which is localized in the synaptic sarcoplasma, has also been shown to decline in level following denervation. Unfortunately, the gene encoding this antigen has not been cloned and therefore, we do not know if it is encoded by INN1.

The mechanism by which INN1 decreases in expression following denervation remains obscure. Because of the paucity of genes whose expression declines after denervation, there has not been any systematic study of the molecular mechanism involved. In theory, two mechanisms could account for such a reduction in expression following denervation. First, the expression of INN1 might be dependent upon the presence of nerve; denervation causes degeneration of nerve terminal and might cause a

loss or reduction of neurotrophic factors that are secreted by the nerve terminal. In this scenario, the neurotrophic factor involved must be different from the signal that activates synapse-specific transcription of AChRs, since this signal has been shown to reside in the synaptic basal lamina and is stable for weeks following denervation (Jo and Burden, 1992). Alternatively, nerve-evoked electrical activity might be critical for the optimal expression of INN1. Loss of electrical activity associated with denervation results in the decline in the expression of INN1. Two experiments should help distinguish between the two possible mechanisms. The spatial distribution of INN1 mRNA should be determined with in situ hybridization, since the first mechanism predicts local accumulation of INN1 mRNA in the synaptic region of the muscle fiber. In addition, denervated muscle could be stimulated by implanted electrodes to provide electric activity, and the effect of such activity on INN1 expression could be determined. If the second mechanism applies, this manipulation should restore the level of expression to that in innervated muscle.

Subtractive hybridization is one of the approaches to detect and identify changes in muscle as a result of denervation. However, this approach is limited in that it can only identify changes in muscle gene expression. It is likely that not all changes in muscle physiology which are affected by electrical activity depend on new protein synthesis or changes in the rate of transcription. For example, muscle activity might alter the half-life of proteins, modify their activity, or affect the cellular localization of proteins. So far, there is not a systematic approach to identify such post-transcriptional modifications of proteins. It is likely, however, that these post-transcriptional mechanisms act coordinately with transcriptional mechanisms. In this respect, it shall be interesting to determine whether the muscle genes identified in the subtraction screen are regulated post-transcriptionally.

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Table 2.1. Subtraction flow chart

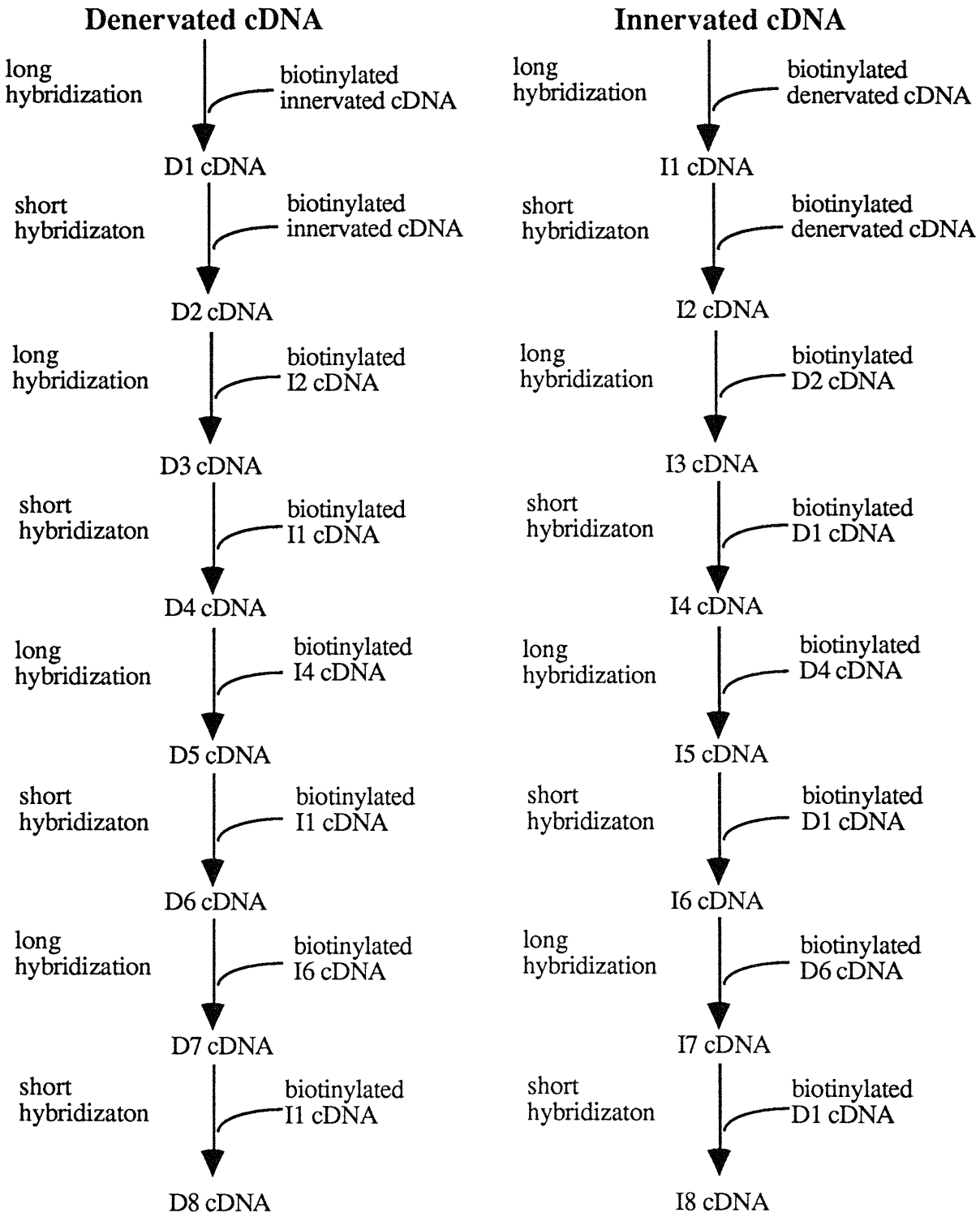


Table 2.2. mRNAs identified by subtractive-hybridization cloning

cDNA	RNA level (Den/Inn)	Abundance (%) in innervated muscle
AChR α subunit	15	0.001
AChR δ subunit	15	0.001
N-CAM	40	0.001
crystallin (αB)	6	0.02
MAP1a	12	0.001
filamin	5	0.04
AML1	100	0.0001
DEN1. Novel, but contains Ig and FN III repeats that are organized like those in titin.	50-100	0.001
DEN2. No homology	10	0.05
INN1. 23 kDa protein that has homology to NSP.	0.1	0.05

Figure 2.1 Complete nucleotide sequence of INN1.

```
5'  GAATTCGGACTGCAGGCTTAGTCTGGGGAAGCGGGTGTTTCATGTCTCAGGGAGAATTTT       60
    GCAGTTTACAGCGTTTCTGTTGGTATGCATAATTTGTAATTGCTGCTGGAGGGCAGATCG      120
    TGGCAAGAA   ATG GAC GGA CAG AAG AAA CAT TGG AAG GAC AAG GTT GTT    168
                 M   D   G   Q   K   K   H   W   K   D   K   V   V   (13)

    GAC CTC CTC TAC TGG AGA GAC ATT AAG AAG ACT GGA GTG GTG TTT GGT    216
     D   L   L   Y   W   R   D   I   K   K   T   G   V   V   F   G   (29)

    GCC AGC TTA TTC CTG CTG CTG TCT CTG ACA GTG TTC AGC ATT GTC AGT    264
     A   S   L   F   L   L   L   S   L   T   V   F   S   I   V   S   (45)

    GTA ACG GCC TAC ATT GCC TTG GCC CTG CTC TCG GTG ACT ATC AGC TTT    312
     V   N   A   Y   I   A   L   A   L   L   S   V   T   I   S   F   (61)

    AGG ATA TAT AAG GGC GTG ATC CAG GCT ATC CAG AAA TCA GAT GAA GGC    360
     R   I   Y   K   G   V   I   Q   A   I   Q   K   S   D   E   G   (77)

    CAC CCA TTC AGG GCA TAT TTA GAA TCT GAA GTT GCT ATA TCA GAG GAA    408
     H   P   F   R   A   Y   L   E   S   E   V   A   I   S   E   E   (93)

    TTG GTT CAG AAA TAC AGT AAT TCT GCT CTT GGT CAT GTG AAC AGC ACA    456
     L   V   Q   K   Y   S   N   S   A   L   G   H   V   N   S   T  (109)

    ATA AAA GAA CTG AGG CGG CTT TTC TTA GTT GAT GAT TTA GTT GAT TCC    504
     I   K   E   L   R   R   L   F   L   V   D   D   L   V   D   S  (125)

    CTG AAG TTT GCA GTG TTG ATG TGG GTG TTT ACT TAT GTT GGT GCC TTG    552
     L   K   F   A   V   L   M   W   V   F   T   Y   V   G   A   L  (141)

    TTC AAT GGT CTG ACA CTA CTG ATT TTA GCT CTG ATC TCA CTC TTC AGT    600
     F   N   G   L   T   L   L   I   L   A   L   I   S   L   F   S  (157)

    ATT CCT GTT ATT TAT GAA CGG CAT CAG GTG CAG ATA GAT CAT TAT CTA    648
     I   P   V   I   Y   E   R   H   Q   V   Q   I   D   H   Y   L  (173)

    GGA CTT GCA AAC AAG AGT GTT AAG GAT GCC ATG GCC AAA ATC CAA GCA    796
     G   L   A   N   K   S   V   K   D   A   M   A   K   I   Q   A  (189)

    AAA ATC CCT GGA TTG AAG CGC AAA GCA GAT TGA AAAAGCCCCAAACAGA       745
     K   I   P   G   L   K   R   K   A   D   *                     (199)
    AGTTCATCTTTAAAGGGGACACTCACTTGATTACGGGGGTGGGAGGGTCAGGGGTGAGCC      805
    CTTGGTGGCCGTGCGGTTTCAGCTCTTTATTTTTAGCAGTGCACTGTTTGAGGAAAAATT      865
    ACCTGTCTTGACTTCCTGTGTTTATCATCTTAAGTATTGTAAGCTGCTGTGTATGGATCT      925
    CATTGTAGTCACACTTGTCTTCCCCAATGAGGCGCCTGGTGAATAAAGGACTCGGGGAAA      985
    GCTGTGCATTGTATCTGCTGCAGGGTAGTCTAGCTGTATGCAGAGAGTTGTAAAGAAGGC     1045
    AAATCTGGGGGCAGGGAAAACCCTTTTCACAGTGTACTGTGTTTGGTCAGTGTAAAACTG     1105
    ATGCAGATTTTTCTGAAATGAAATGTTTAGATGAGAGCATACTACTAAAGCAGAGTGGAA     1165
    AACTCTGTCTTTATGGTGTGTTCTAGGTGTATTGTGAATTTACTGTTATATTGCCAATAT     1225
    AAGTAAATATAGACCTAATCTATATATAGTGTTTCACAAAGCTTAGATCTTTAACCTTGC     1285
    AGCTGCCCCACAGTGCTTGACCTCTGAGTCATTGGTTATGCAGTGTAGTCCCAAGCACAT     1345
    AAACTAGGAAGAGAAATGTATTTGTAGGAGTGCTACCTACCACCTGTTTTCAAGAAAATA     1405
    TAGAACTCCAACAAAAATATAGAATGTCATTTCAAAGACTTACTGTATGTATAGTTAATT     1465
    TTGTCACAGACTCTGAAATTCTATGGACTGAATTTCATGCTTCCAAATGTTTGCAGTTAT     1525
    CAAACATTGTTATGCAAGAAATCATAAAATGAAGACTTATACCATTGTGGTTTAAGCCGT     1585
    ACTGAATTATCTGTGGAATGCATTGTGAACTGTAAAAGCAAAGTATCAATAAAGCTTATA     1645
    GACTT(A)20  3'                                                   1650
```

Figure 2.2. Northern blot analysis of DEN1 mRNA in innervated and denervated leg muscle.

The level of DEN1 mRNA is undetectable in innervated muscle, and its level rises substantially following denervation. One single transcript of 8 kb is detected in denervated muscle.

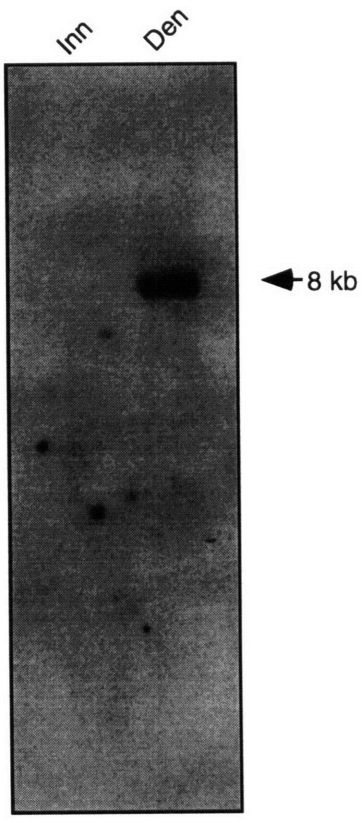

Inn
Den
8 kb

Figure 2.3. INN1 is expressed in a variety of tissues.
The abundance of INN1 mRNA in various tissues is measured by RNase protection. GAPDH mRNA level is also determined by RNase protection to normalize the amount of RNA. INN1 expression is detected in nearly all tissues examined, except for liver, which shows a abnormally low level of GAPDH mRNA.

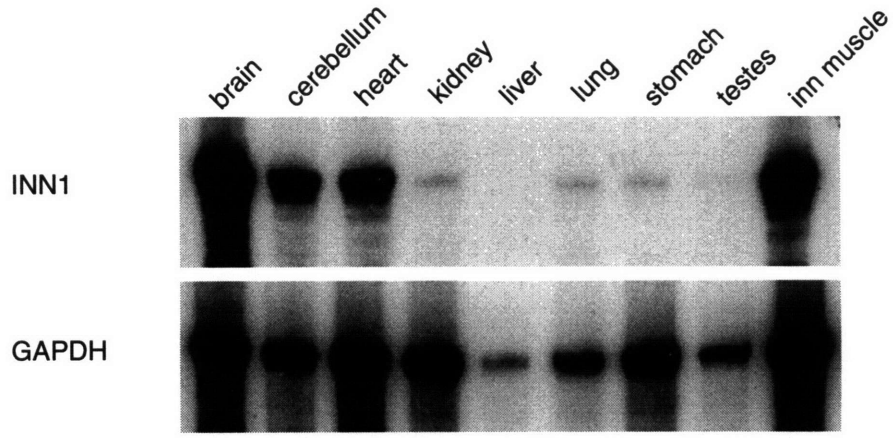
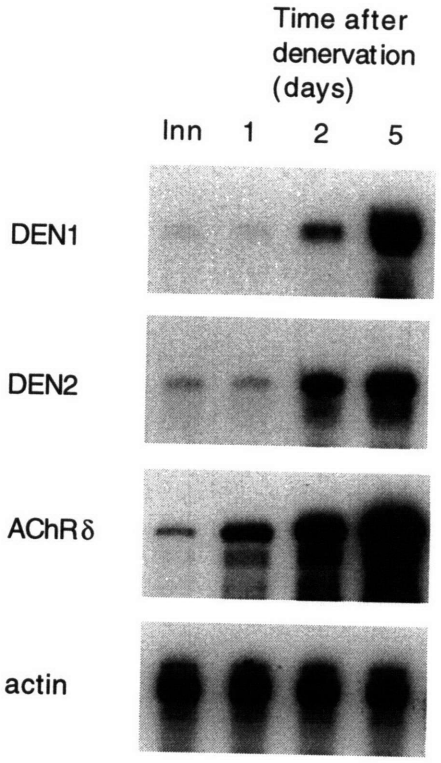

brain
cerebellum
heart
kidney
liver
lung
stomach
testes
inn muscle
INN1
GAPDH

Figure 2.4. Expression of DEN1 and DEN2 genes increases with a slow time course after denervation.

Rat lower leg muscles were denervated for 1, 2, and 5 days, and the levels of DEN1, DEN2, AChR δ subunit and actin mRNAs were measured by RNase protection assay. Inn, innervated muscle.


Time after
denervation
(days)
Inn
1
2
5
DEN1
DEN2
AChRδ
actin

Chapter 3

AML1 Is Expressed in Skeletal Muscle and Is Regulated by Innervation

The work described in this chapter has been published in:
Zhu, X., Yeadon, J. E., and Burden, S. J. (1994) AML1 is expressed in skeletal muscle and is regulated by innervation. *Mol. Cell. Biol.* **14**, 8051-8057.

SUMMARY

Although most skeletal muscle genes are expressed at similar levels in electrically active, innervated muscle and in electrically inactive, denervated muscle, a small number of genes, including those encoding the acetylcholine receptor, N-CAM and myogenin, are expressed at significantly higher levels in denervated than in innervated muscle. The mechanisms that mediate electrical activity-dependent gene regulation are not understood, but these mechanisms are likely to be responsible, at least in part, for the changes in muscle structure and function that accompany a decrease in myofiber electrical activity. To understand how muscle activity regulates muscle structure and function, I used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. One of the genes which we found to be regulated by electrical activity is the recently discovered acute myeloid leukemia 1 (AML1) gene. Disruption and translocation of the human AML1 gene is responsible for a form of acute myeloid leukemia. AML1 is a DNA binding protein, but its normal function is not known and its expression and regulation in skeletal muscle was not previously appreciated. Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the AML1 gene in innervated, denervated and developing skeletal muscle. We show that AML1 is expressed at low levels in innervated skeletal muscle and at 50 to 100-fold greater levels in denervated muscle. Four AML1 transcripts are expressed in denervated muscle, and the abundance of each transcript increases after denervation. We transfected C2 muscle cells with an expression vector encoding AML1, tagged with an epitope from hemagglutinin, and we show that AML1 is a nuclear protein in muscle. AML1 dimerizes with core binding factor β (CBF β), and we show that CBF β is expressed at high levels both in innervated and denervated skeletal muscle. PEBP2α, which is structurally related to AML1 and which also dimerizes with CBF β, is expressed at low levels in skeletal muscle and is up-regulated only weakly by denervation. These results are consistent with the idea that AML1 may have a role in regulating gene expression in skeletal muscle.

INTRODUCTION

Vertebrate neuromuscular synapses arise as a result of complex interactions between motor neurons and developing skeletal muscle cells (Hall and Sanes, 1993). One of the more striking specializations of the neuromuscular synapse is the remarkable concentration of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane (Hall and Sanes, 1993; Jennings and Burden, 1993). Experiments designed to determine how AChR expression is regulated have revealed that a combination of three separate

mechanisms mediate the clustering of AChRs at synaptic sites. First, motor neurons synthesize and deposit agrin in the synaptic basal lamina, where it induces a redistribution of non-synaptic AChRs to the newly formed synapse (Anderson and Cohen, 1977; McMahan, 1990; Nastuk and Fallon, 1993). Second, an unknown factor, which like agrin is deposited into the synaptic basal lamina and which is presumably synthesized by motor neurons, activates a signaling pathway leading to transcription of AChR genes selectively in nuclei near the synaptic site (Burden, 1993). Finally, nerve-induced muscle electrical activity represses AChR transcription throughout the muscle fiber, decreasing the number of AChRs expressed in the non-synaptic region (Hall and Sanes, 1993; Jennings and Burden, 1993).

The molecular mechanisms that mediate electrical activity-dependent regulation of AChR genes are unknown, but a clearer view of the electrical activity-dependent signaling pathway is beginning to emerge (Huang et. al, 1992; Laufer and Changeux, 1989; Tang et al., 1994). Recent studies demonstrate that a binding site (E-box) for myogenic basic helix-loop-helix (bHLH) proteins in the AChR δ subunit gene is critical for activity-dependent gene regulation, and these results suggest that an E-box binding protein(s) is directly involved in electrical activity-dependent regulation of the δ subunit gene (Tang et al., 1994). Because the level of mRNAs encoding myogenic bHLH proteins rises substantially following denervation (Duclert et al., 1991; Eftimie, et al., 1991; Neville et al., 1992; Voytik et al., 1993; Witzemann and Sakmann, 1991), these proteins may directly mediate the increase in AChR expression in denervated muscle by binding to the AChR regulatory region (Jennings and Burden, 1993). Nevertheless, electrical activity-dependent regulation of the AChR subunit genes may be controlled by transcription factors in addition to E-box binding proteins, and expression of other genes that are regulated by electrical activity may be controlled by other pathways and transcription factors. Thus, it remains unclear whether E-box binding proteins are the only transcriptional mediators of electrical activity-dependent regulation.

In addition to its role in repressing AChR gene expression, nerve-induced muscle activity has been implicated as a regulator of other steps in the formation and maturation of synapses. For example, motor neuron survival, motor neuron growth and editing of initial synaptic connections are affected by nerve-induced muscle activity (Hall and Sanes, 1993). To understand how muscle activity regulates muscle structure and function, we used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. We identified several genes that are expressed preferentially in denervated muscle and one gene that is expressed preferentially in innervated muscle. One of the genes that we found to be regulated by

electrical activity is the recently discovered acute myeloid leukemia 1 (AML1) gene (Miyoshi et al., 1993).

Disruption and translocation of the human AML1 gene is responsible for a form of acute myeloid leukemia (Erickson et al., 1992; Miyoshi et al., 1993). AML1 is a DNA-binding protein, containing a DNA-binding region homologous to the Drosophila runt protein (Bae et al., 1994; Daga et al., 1992; Erickson et al, 1992; Kania et al., 1990). The normal function of AML1, however, is not known and its expression and regulation in skeletal muscle was not previously appreciated. AML1 is thought to dimerize with core binding factor β (CBF β) (Ogawa et al., 1993a; Ogawa et al, 1993b; Wang et al., 1993), and translocations in the β subunit gene likewise cause acute myeloid leukemia (Liu et al., 1993). The AML1/CBF β heterodimer was purified independently on the basis of its ability to bind core sequences (Speck and Baltimore, 1987; Wang and Speck, 1992), which are important for viral pathogenesis, in murine leukemia virus enhancers (Hallberg et al, 1991; Speck et al., 1990). Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the AML1 gene in innervated, denervated and developing skeletal muscle.

MATERIALS AND METHODS

RNA Analysis

RNase protection assays were performed as described previously (Wang et al., 1993). Briefly, antisense RNA was hybridized with total RNA (Chomczynski and Sacchi, 1987) in hybridization buffer overnight at 42°C. Non-hybridized RNA was digested with RNase (A and T1) for 1 hour at 30°C. Following proteinase K digestion and phenol extraction, the protected RNA was precipitated by ethanol and fractionated in 5% denaturing polyacrylamide gels.

mRNA was purified by oligo-dT cellulose chromatography (Aviv and Leder, 1972), electrophoresed in a 1% formaldehyde agarose gel and transferred to a GeneScreen (Du Pont) membrane. DNA probes were labelled by random-hexamer-priming. The membranes were hybridized overnight in 5XSSPE, 5X Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA at 68°C. The filter was washed in 0.1% SDS, 0.1XSSPE for 1 hour at 68°C and exposed for 2 days with an intensifying screen. Northern blots and RNase protection assays were quantitated with a PhosphorImager.

Epitope-tagging of AML1

The hemagglutinin (HA) epitope-tag was added to the carboxyl terminus of AML1 by PCR. The HA-tagged AML1 cDNA was inserted into a myosin light chain vector (MDAF, kindly provided by Dr. J. P. Merlie). Mouse C2 cells were grown and transfected as described previously (48). Pooled stably transfected cells were stained with a monoclonal antibody (9E10) against the HA epitope; the 9E10 hybridoma cell line was obtained from ATCC.

Site-directed mutagenesis

The AChR δ subunit 5' regulatory sequence, -840/+25, was mutagenized by site-directed mutagenesis (Simon and Burden, 1993). The mutations were confirmed by sequencing, and the mutated regulatory sequence was subcloned into p0hGH (Selden et al., 1986). AChR δ-hGH and pSV2-CAT plasmids were co-transfected into C2 myoblasts, the amount of hGH secreted from myotubes was determined by a radioimmunoassay 4 or 5 days after transfection and the amount of secreted hGH was normalized to the level of CAT activity (Simon and Burden, 1993). Duplicate dishes were included in each experiment; expression from duplicate dishes varied by less than 20%.

Electrophoretic mobility -shift assay

AML1-binding to wild-type and mutated sequences was assayed by an electrophoretic mobility-shift assay (Wang et al., 1993). Oligonucleotides were end-labelled and purified with a Biospin-3 column (Biorad), and AML1/CBF β proteins were translated in reticulocyte lysates (Promega).

The GenBank/EMBL accession number for the rat AML1 sequence is L35271.

RESULTS

AML1 expression increases following denervation

We isolated several cDNA fragments encoding the rat homologue of human AML1 from our denervated minus innervated muscle library, and we used the cDNA fragment to isolate a cDNA encoding full-length AML1 protein from a rat skeletal muscle library. The 1.8 kbp cDNA contains an ORF of 1.35 kbp that encodes a 48.5 kd protein which is highly homologous to human (99%) and mouse (99%) AML1 (Bae et al., 1993) and less homologous (82%) to the related mouse protein, PEBP2α (Ogawa et al., 1993b). The rat cDNA encodes an AML1 protein that is identical in length to the mouse AML1 protein but longer than the human AML1 protein.

To confirm that AML1 expression is regulated by innervation, we measured the level of AML1 mRNA in innervated and denervated muscle by an RNase protection assay. Figure 1 shows that AML1 is expressed in rat skeletal muscle and that AML1 expression increases substantially (50 to 100-fold) following denervation.

Because Schwann cells and other perisynaptic cells respond to denervation (Brocks, 1984; Gatchalian, et al., 1989; Reynolds and Woolf, 1992), we considered the possibility that perisynaptic cells, rather than muscle cells, accounted for the increased AML1 expression in denervated muscle. Muscle can be conveniently dissected into a synaptic region, containing perisynaptic cells, and a non-synaptic region, lacking perisynaptic cells (Merlie and Sanes, 1985). We dissected the synaptic and non-synaptic regions of innervated and denervated skeletal muscle and measured the level of AML1 expression in each region. Figure 2 shows that AML1 expression in the non-synaptic region increases 50 to 100-fold following denervation. Because non-synaptic regions of muscle lack Schwann cells and other perisynaptic cells, this result indicates that muscle fibers are the predominant source of AML1 RNA in denervated muscle.

Although AML1 is expressed at similar levels in synaptic and non-synaptic regions of innervated muscle, AML1 is expressed preferentially in the synaptic region of denervated muscle (Fig. 2). We do not know the cellular source of the preferential AML1 expression in the synaptic region of denervated muscle; AML1 expression may be greater in the synaptic than the non-synaptic region of denervated muscle fibers and/or AML1 expression may also increase in Schwann cells or other perisynaptic cells following denervation.

The distribution of AML1 expression in different tissues has received little attention. Both B-cell lines and T-cell lines express multiple AML1 transcripts (2.1, 4.3, 5.4, 8.2 kb), although the rank order of their abundance differs among different cell lines (Bae et al., 1993; Miyoshi et al., 1991). Although little is known about the different transcripts, there is evidence that the different transcripts encode different proteins (Bae et al., 1994). Four AML1 transcripts, which are similar in size to those in B-cell lines and T-cell lines, are expressed in denervated skeletal muscle, and the abundance of each transcript increases following denervation (Fig. 3). The predominant AML1 transcripts in denervated skeletal muscle are ~7.0 and 6.3 kb long.

We measured the time course of AML1 expression following denervation using an RNase protection assay. A two-fold increase in AML1 expression is detectable by 1 day after denervation, and a 50-100 fold increase is detectable by 5 days after denervation (Fig. 4). The increases in AChR and N-CAM expression following denervation follow a similar time course (Covault et al., 1987; Merlie et al., 1984), whereas the increase in

myogenin expression precedes that of the other genes (Duclert et al., 1991; Eftimie et al., 1991; Neville et al., 1992; Voytik et al., 1993; Wilson et al., 1984).

Although most skeletal muscle genes are expressed at similar levels in fast and slow muscle fibers, a subset of muscle genes are expressed preferentially in certain fiber types (Atomi et al., 1991; Donoghue et al., 1991; Hughes et al., 1993; Pette and Staron, 1990; Schiaffino et al., 1989; Voytik, et al., 1993). To determine whether AML1 is expressed preferentially in fast or slow myofibers, we measured the abundance of AML1 RNA in innervated and denervated rat soleus (slow) and extensor digitorum longus (EDL) (fast) muscles. Figure 5 shows that AML1 is expressed at similar levels in rat soleus and EDL muscles and that denervation causes a 50 to 100-fold increase in AML1 expression in both slow and fast muscles. Thus, the regulation of AML1 expression is similar in slow and fast muscles.

Many genes that are expressed in skeletal muscle are expressed at low levels in myoblasts and at significantly higher levels following myoblast fusion and muscle differentiation. To determine whether AML1 is induced during muscle differentiation, we measured AML1 expression in C2 myoblasts and myotubes. Figure 6 shows that expression of AML1 is not up-regulated during muscle differentiation. Indeed, the level of AML1 expression is similar in myoblasts, embryonic myotubes and denervated adult muscle (Fig. 6).

CBF β and PEBP2α mRNAs are expressed in skeletal muscle

AML1 is thought to complex with a second protein, termed CBF β, and the heterodimer, CBF, has a higher affinity for an AML1 binding site (see below) than AML1 alone (Ogawa et al., 1993a; Ogawa et al., 1993b; Wang et al., 1993). Therefore, we determined whether CBF β is expressed in skeletal muscle. Figure 7 shows that CBF β RNA is expressed in skeletal muscle (Wang et al., 1993). CBF β is more abundant than AML1 in innervated muscle, but CBF β expression increases little following denervation. As a consequence, CBF β and AML1 are expressed at similar levels in denervated muscle.

PEBP2α is structurally related to AML1 and contains a runt homology region (Ogawa et al., 1993b), but its expression is thought to be restricted to T cells, where it can function as a transcriptional activator (Ogawa et al., 1993b). To determine whether PEBP2α is expressed in skeletal muscle, we measured the level of PEBP2α RNA in innervated and denervated muscle. Figure 7 shows that PEBP2α is expressed at low levels in innervated skeletal muscle and at ~4-fold greater levels following denervation.

CBF was purified from thymus nuclear extracts on the basis of its ability to bind core sites in viral enhancers (Kamachi et al., 1990; Wang and Speck, 1993). To

determine whether AML1 is a nuclear protein in skeletal muscle, we transfected an expression vector encoding AML1, tagged with an epitope from HA, into C2 muscle cells and located the epitope-tagged protein by immunofluorescence. Figure 8 shows that AML1 expression is restricted to the nuclei of myoblasts and myotubes. Thus, AML1 is a nuclear protein in muscle.

The cis-acting region of the AChR δ subunit gene that confers regulation by electrical activity lacks a functional binding site for AML1/CBF β

A consensus binding site for CBF has been determined by site-selection assays (Melnikova et al., 1993). The regulatory region of the AChR δ subunit gene, which confers innervation-dependent regulation (Tang et al., 1994), lacks this consensus sequence but contains a sequence which deviates from the consensus sequence by a single nucleotide.

We used recombinant AML1/CBF β, synthesized *in vitro*, to determine whether this variant sequence binds AML1. Figure 9 shows that AML1 and AML1/CBF β bind the variant sequence in the δ subunit regulatory region but with a 30-fold lower affinity than the consensus CBF target sequence.

To determine whether the weak AML1-binding sequence is required for δ subunit gene expression, the AML1-binding site (AACCACC) in the AChR δ-subunit regulatory region (positions -31 to -25) was mutagenized to generate either a nonfunctional (AACGTCC) or a consensus (AACCACA) binding sequence for AML1 (Melnikova et al, 1993). Gene fusions between hGH and wild-type or mutated AChR δ-subunit sequences (nucleotides -840 to +25) were transfected into C2 myoblasts, and hGH expression was measured as described in Materials and Methods. We found that a mutation which prevents AML1/CBF β binding has little or no effect on δ-subunit expression in cultured myotubes. Moreover, mutation of the weak AML1-binding sequence to a consensus AML1-binding sequence has little or no effect on δ subunit expression. These results indicate that the weak AML1-binding sequence is not a target for AML1-mediated transcriptional activation in myotubes grown in cell culture.

DISCUSSION

We show that AML1 is expressed in skeletal muscle and is regulated by electrical activity. Although previous studies had shown that AML1 is expressed in 3T3 cells, B-cell lines and T-cell lines, expression of AML1 in other cell types and tissues had not been examined. Our results demonstrate that AML1 expression is not restricted to the

hematopoetic lineage; rather, AML1 is expressed in skeletal muscle where it is regulated by physiological signals.

Alternative splicing of AML1 RNA results in four transcripts of different sizes. We found that the level of all four AML1 transcripts increases in response to denervation. Although little is known about the different transcripts, the different transcripts encode different proteins which may have different functions (Bae et al., 1994; Bae et al., 1993; Meyers et al., 1993; Miyoshi et al., 1991). Indeed, others have speculated that certain isoforms of AML1 may bind DNA and activate transcription whereas other isoforms may bind DNA but repress gene expression (Meyers et al., 1993).

Since translocations involving either AML1 or the CBF β subunit gene lead to leukemia, one might predict that AML1 might participate in regulation of cell growth or differentiation. Our data, however, does not support the idea that AML1 regulates proliferation during muscle differentiation, since AML1 is expressed at similar levels in both proliferating myoblasts and in differentiated myotubes grown in cell culture. Our studies, however, do not address the possibility that AML1 might be regulated post-transcriptionally during muscle differentiation or that AML1 might regulate muscle growth later in development.

It is not clear how the t(8;21) DNA rearrangement, which results in a fusion between the AML1 and ETO (eight twenty one) genes, causes leukemia. Since AML1 can bind to target sequences in the T-cell receptor β enhancer and activate transcription (Bae et al., 1994), it is possible that the gene fusion disrupts the normal transcriptional activity of AML1 and that the function of wild-type AML1 is inhibited by the mutant AML1 protein in a dominant negative manner (Bae et al., 1994; Miyoshi et al., 1993). Alternatively, the gene fusion could result in a novel transcriptional activity, which in turn leads to aberrant gene expression and cell proliferation.

Some of the muscle genes whose expression is repressed by electrical activity, such as AChR subunit genes and N-CAM, are also locally expressed or concentrated in the synaptic region. At present, we do not know the distribution of AML1 protein or mRNA within the muscle fibers. Our experiment to examine the localization of AML1 mRNA by measuring its abundance in dissected synapse-rich and synapse-free muscle fractions does not provide evidence for the enrichment of AML1 mRNA in the synaptic region. However, our dissection was relatively crude, the synaptic-rich region we obtained still contains a vast number of non-synaptic nuclei; therefore, even a very low level of AML1 expression in the non-synaptic region would mask the mRNA concentration gradient. A more careful study with in situ hybridization will be necessary to determine the distribution of AML1 mRNA in muscle.

Muscle genes that are regulated by electrical activity, including those that regulate muscle atrophy, motor neuron survival, motor neuron growth and editing of initial synaptic connections are potential targets for AML1. Our data, however, demonstrate that the AChR δ subunit gene is not directly regulated by AML1 in developing muscle, and these results suggest that AML1 does not directly regulate expression of the AChR δ subunit gene in response to changes in muscle fiber electrical activity. If AML1 has a role in electrical activity-dependent regulation of the AChR δ subunit gene, then AML1 is likely to act indirectly. The target genes for AML1 and the consequences of increased AML1 expression in muscle may be best examined by increasing AML1 expression in innervated muscle or decreasing AML1 expression in denervated muscle.

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Figure 3.1. AML1 expression increases 50 to 100-fold following denervation.

RNA was extracted from 5-day denervated (Den) or contralateral innervated (Inn) rat lower leg muscle, and the levels of AML1 and actin mRNAs were measured by RNase protection. The level of AML1 mRNA increases approximately 50 to 100-fold after denervation. Actin mRNA level remains unchanged.

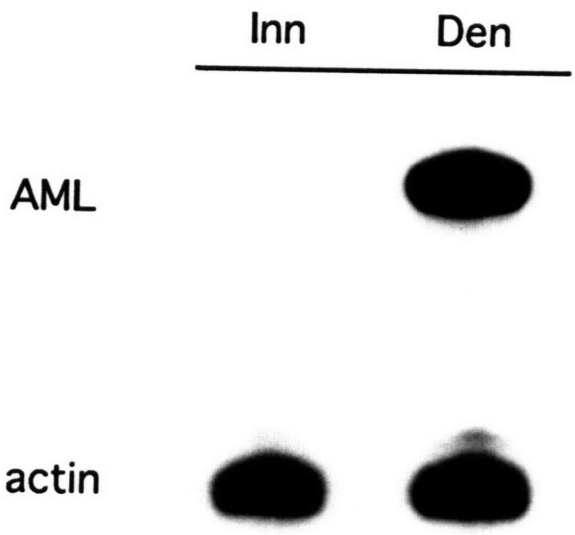

Inn
Den
AML
actin

Figure 3.2. AML1 expression increases in non-synaptic regions of rat soleus muscle following denervation.

Innervated (Inn) and 5-day denervated (Den) soleus muscle were dissected into synaptic (ep+) and non-synaptic (ep-) regions, and the levels of AML1 and actin mRNAs in each region were measured by RNase protection. AML1, normalized to actin, is expressed at similar levels in synaptic and non-synaptic regions of innervated muscle; the synaptic region contains 10-fold more AChR ε subunit mRNA than the non-synaptic region. AML1 is expressed at 5-fold higher levels in the synaptic than non-synaptic region of denervated muscle.

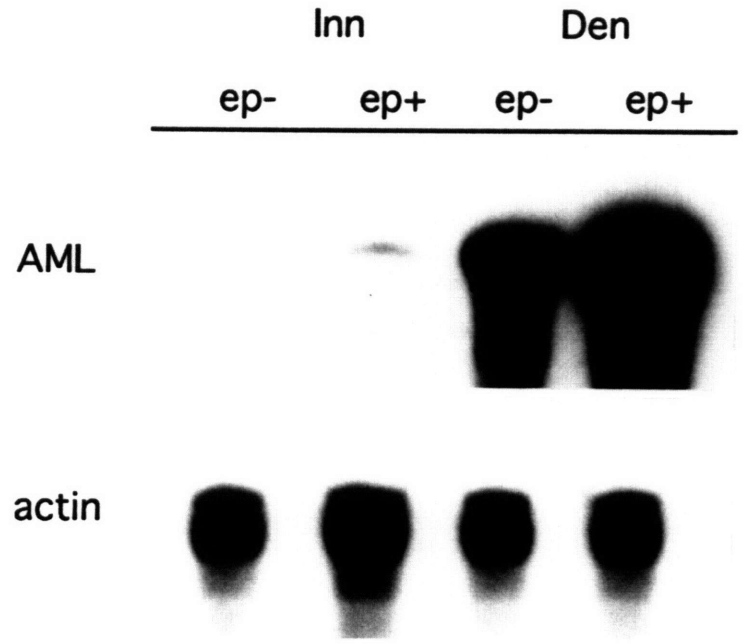

Inn
Den
ep-
ep+
ep-
ep+
AML
actin

Figure 3.3. The level of four AML1 transcripts increases following denervation.

Innervated and denervated rat muscle mRNA (3 μg) were fractionated by gel electrophoresis and transferred to a GeneScreen membrane. The filter was hybridized with a full-length AML1 cDNA probe and exposed for 2 days with an intensifying screen. Four transcripts (arrowheads), 7.0, 6.3, 3.9 and 3.4 kb in size, are detected in denervated muscle but not innervated muscle. The level of actin mRNA in innervated and denervated muscle was similar.

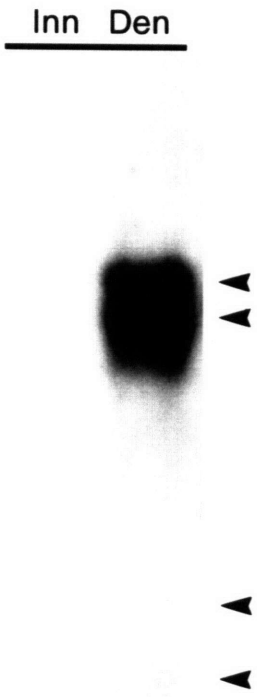

Inn Den

Figure 3.4. Expression of AML1 and AChR genes increase with a similar time course after denervation.

Rat lower leg muscles were denervated for 1, 2 or 5 days, and the levels of AML1, AChR δ subunit, myogenin and actin mRNAs were measured by RNase protection. AML1 expression increases approximately two-fold one day after denervation, and increases 50- to 100-fold 5 days after denervation. AChR δ subunit expression increases in parallel following denervation. Myogenin expression, in contrast, reaches its highest level after 2 days and therefore, precedes that of AML1 and the AChR δ subunit gene.

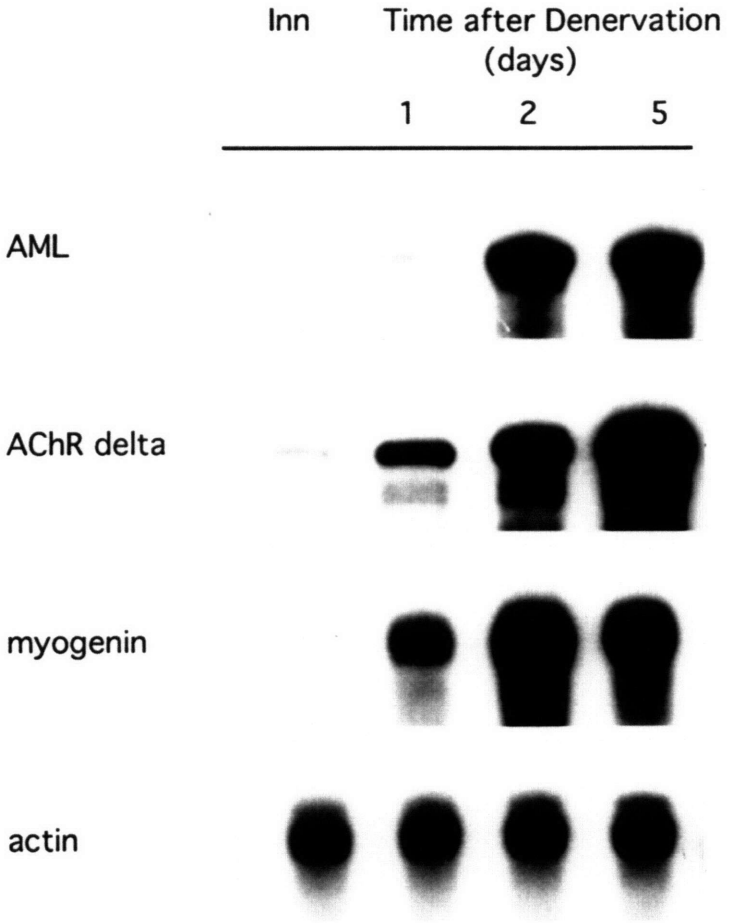

Inn
Time after Denervation
(days)
1
2
5
AML
AChR delta
myogenin
actin

Figure 3.5. AML1 expression increases in both slow (soleus) and fast (EDL) muscle after denervation.

The level of AML1 and actin mRNA in innervated and denervated soleus and EDL muscles were quantitated by RNase protection. The level of AML1 expression is comparable in denervated slow (soleus) and fast (EDL) muscles.

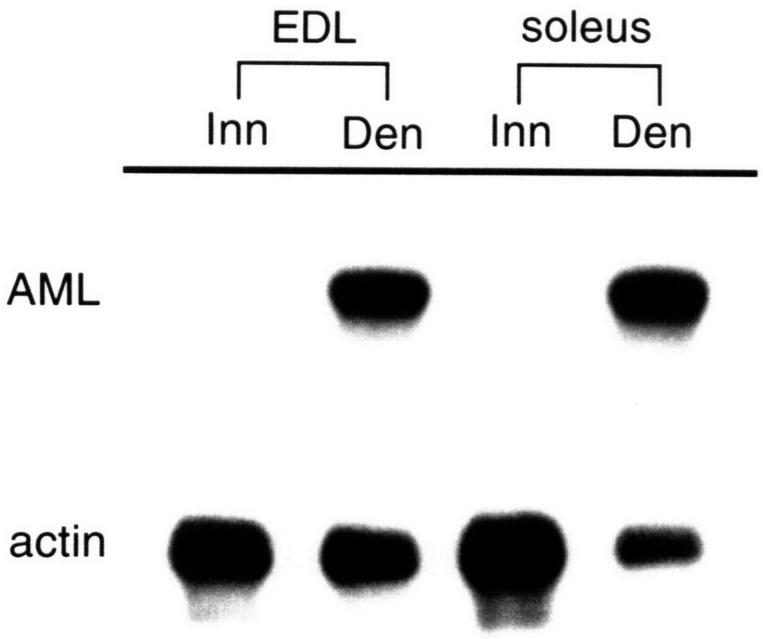

EDL
soleus
Inn
Den
Inn
Den
AML
actin

Figure 3.6. AML1 is expressed at similar levels in cultured myoblasts and myotubes.

Total RNA was extracted from cultured C2 myoblasts (MB) and myotubes (MT), and the level of AML1 and actin mRNAs was quantitated by RNase protection. The level of AML1 mRNA is comparable in C2 myoblast and myotubes.

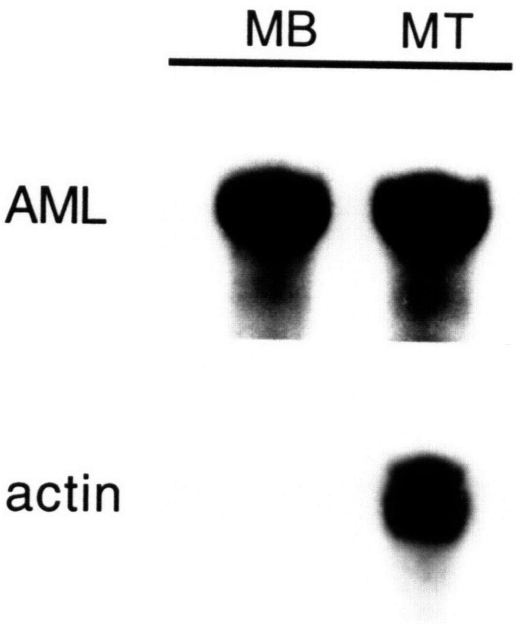

MB
MT
AML
actin

Figure 3.7. CBF β and PEBP2α are expressed in skeletal muscle.
CBF β expression increases ~2-fold and PEBP2α expression increases ~4-fold following denervation. Total RNA was extracted from innervated and denervated (4-day) mouse lower leg muscle, and the level of CBF β and PEBP2α mRNAs were measured by RNase protection.

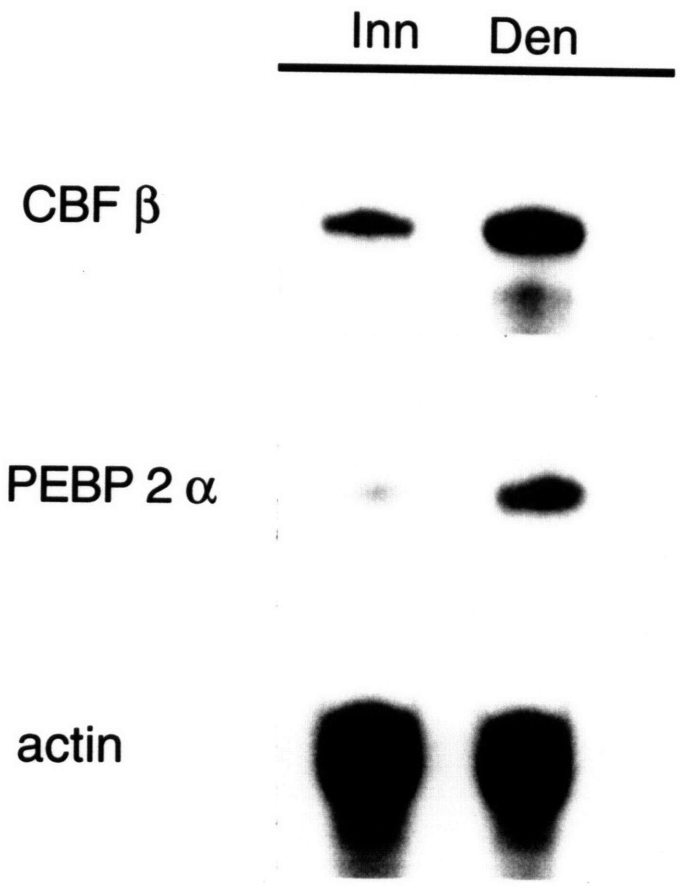

Inn
Den
CBF β
PEBP 2 α
actin

Figure 3.8. AML1 is a nuclear protein in muscle.

HA-tagged AML1 and pSV2 neo plasmid DNAs were co-transfected into C2 myoblasts, and stably transfected cells were selected with G418 (750 μg/ml). Resistant cells were pooled, induced to differentiate into myotubes and stained with a monoclonal antibody (9E10) against the HA epitope (a). Nontransfected C2 myotubes are not stained by the monoclonal antibody 12CA5 (b).

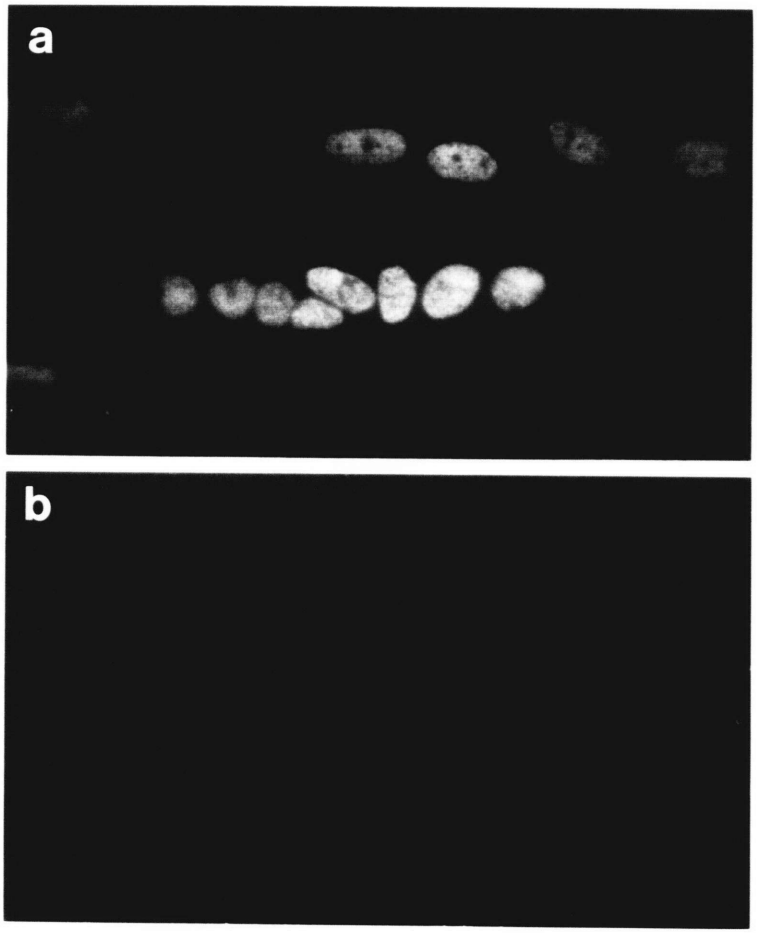
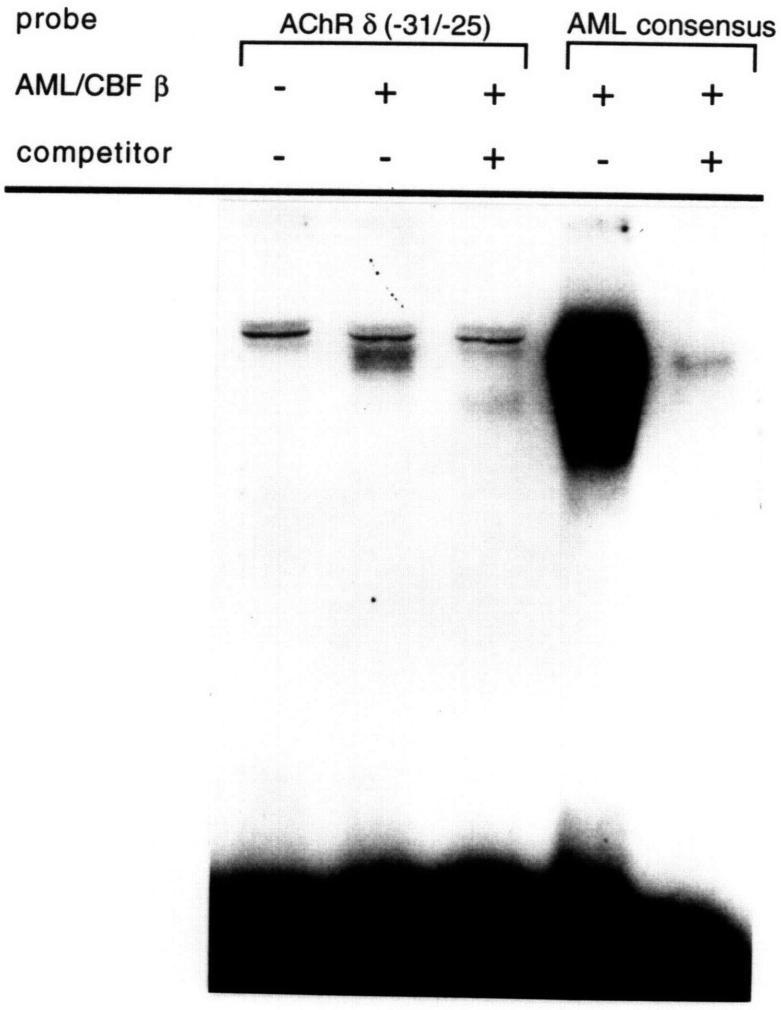

a
b

Figure 3.9. AML1/CBF β binds weakly to a sequence in the AChR δ subunit regulatory region.

AML1/CBF β proteins were translated *in vitro* and incubated with a consensus binding sequence for AML1 (AACCACA) or with a similar sequence (AACCACC) present in the AChR δ subunit regulatory region (-31/-25). 100-fold excess of unlabelled oligonucleotides were used in competition experiments. The consensus sequence has a 30-fold higher affinity for AML1/CBF β than the -31/-25 sequence in the AChR δ subunit regulatory region.


probe
AChR δ (-31/-25)
AML consensus
AML/CBF β
- + + + +
competitor
- - + - +

Chapter 4

Neuregulin Is a Potential Extracellular Signal that Activates Synapse-Specific Gene Expression

Part of the work described in this chapter has been published in:
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SUMMARY

Nicotinic acetylcholine receptors (AChR) are highly concentrated in the synaptic region of skeletal muscle fibers and this localization at synaptic sites is a hallmark of postsynaptic differentiation. Since the only distinction between the synaptic and non-synaptic region of the muscle fiber is its close contact with motor nerve terminal, localization of AChRs at synapses is thought to be induced by motor neurons. It is now believed that the motor nerve induces synaptic accumulation of AChRs by two independent pathways. In the first pathway, the motor neuron secretes agrin, which is incorporated into the synaptic basal lamina and which clusters pre-existing AChRs to the synaptic region of the muscle fiber. In the second pathway, an unknown molecule secreted by the motor nerve instructs muscle synaptic nuclei to transcribe AChR subunit genes at a higher rate than non-synaptic nuclei. We have attempted to identify the extracellular signaling molecule that activates AChR genes in synaptic nuclei. We show that neuregulin, a protein isolated from chick brain which stimulates AChR synthesis in cultured chick myotubes, is capable of activating AChR δ subunit gene transcription in cultured mouse myotubes, and that the same 181 bp cis-element that confers synapse-specific gene transcription in transgenic mice is sufficient to mediate the response to neuregulin. Moreover, we show by immunostaining that neuregulin is present at neuromuscular synapses and that, like the extracellular signal that stimulates synapse-specific gene expression, neuregulin remains at synaptic sites in the absence of muscle and nerve. In addition, putative receptors for neuregulin, erbB3 and erbB4, are expressed in muscle and are concentrated at synaptic sites. These results suggest strongly that neuregulin is an extracellular signaling molecule that induces synapse-specific transcription of AChR genes.

INTRODUCTION

The neuromuscular synapse is a highly specialized structure formed as a result of inductive interactions between muscle and motor neurons (Hall and Sanes, 1993). The presynaptic terminal is characterized by a high density of synaptic vesicles and active zones, the sites where synaptic vesicles are docked and thought to be released. The postsynaptic specializations include highly involuted junctional folds, and accumulation of several molecules, including AChR, sodium channels and the 43 kd protein (Hall and Sanes, 1993.) Among these, nicotinic AChRs are the best studied, and the mechanism that leads to their localization at synaptic sites has served as a paradigm for studying the formation of the postsynaptic apparatus.

AChRs at mature mammalian neuromuscular junctions are pentameric complexes consisting of four different subunits, $\alpha_2\beta\varepsilon\delta$ (Gu and Hall, 1988). During neuromuscular development, AChRs undergo a maturation process in which the ε subunit replaces the γ subunit. It is believed that this subunit substitution accounts for the change in electrophysiological properties of AChR channels, from low-conductance, long open time to high-conductance, short open time (Mishina et al., 1986). The appearance of AChR ε subunit at synapses is dependent upon motoneuron innervation. Denervation of mouse muscle 4 days after birth prevents the appearance of ε subunit at synapses (Martinou et al., 1991), suggesting that a nerve-derived factor is essential for the initial appearance of AChR ε subunit at synapses.

At mature neuromuscular synapses, which comprises only 0.1% of the total muscle surface, AChRs are present at a density of 10,000 molecules per μm^2, whereas in the extrasynaptic region just 20 μm away from the synapse, the density drops precipitously to approximately 10 molecules per μm^2 (Salpeter and Harris, 1983; Salpeter et al., 1988). Such a high density of AChRs is functionally important since it ensures rapid and efficient synaptic transmission. The molecular mechanisms responsible for localizing AChRs at synapses is a central problem in studies of synapse formation.

It is now believed that AChRs become concentrated at the synapses as a result of two separate processes: aggregation of pre-existing receptors to synaptic sites and local synthesis and insertion of AChRs into the synaptic region of the muscle fiber (Jennings and Burden, 1993).

An understanding of the first process is emerging. Results from many experiments support the theory that agrin, a protein released by motor neurons and subsequently incorporated into the synaptic basal lamina, is responsible for clustering pre-existing AChRs to the synaptic sites (McMahan, 1990). The evidence for the role of agrin in clustering AChRs to synapses is several-fold. First, agrin is present and localized in the basal lamina at the neuromuscular synapse (Reist et al., 1987). Second, agrin is synthesized by motor neurons and is transported to the nerve terminal (Magill-Solc and McMahan, 1988, 1990). Third, in addition to AChRs, agrin is capable of clustering other molecules known to be concentrated at the synapse, including acetylcholine esterases (AChE), a heparin sulfate proteoglycan and the 43 kd protein (Wallace et al., 1985; Nitkin et al., 1987; Wallace, 1986, 1989). Fourth, antibodies that inhibit the aggregation effect of agrin block the appearance of AChR clusters in motor neuron-muscle co-cultures (Reist et al., 1992). The molecular mechanism by which agrin clusters AChRs is not known, but it has been shown that agrin does not affect the synthesis of AChRs (Nitkin et al., 1987), and its effect is not dependent upon ongoing protein synthesis (Wallace, 1989).

Indirect evidence for selective transcription of AChR genes by synaptic nuclei is provided by the observation that AChR mRNAs are concentrated in the synaptic region of the muscle fiber (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). Subsequent studies with transgenic mice demonstrated that selective transcription of AChR subunit genes is, at least in part, responsible for concentrating AChR mRNA in the synaptic region of muscle fiber (Sanes et al, 1991; Simon et al., 1992). In one of these experiments, a 1.8 kb upstream regulatory sequence of AChR δ subunit gene was fused to a reporter gene, human growth hormone (hGH), and introduced into mice (Simon et al., 1992). hGH expression was found to be restricted to the synaptic muscle nuclei, indicating that the 1.8 kb control sequence is sufficient to confer synapse-specific transcription of the AChR δ subunit gene. Furthermore, when both muscle and nerve were damaged and the nerve was prevented from reinnervating the muscle, it was found that hGH staining in the regenerated, degenerated myotubes reappeared at the original synaptic site. Since the basal lamina is the only structure that remains at the original synapse following damage to nerve and muscle, this result indicates that the synaptic basal lamina contains a signal that is capable of activating the AChR δ gene and that this signal is stable throughout the denervation-regeneration period. In a similar study, a gene fusion between a 3.2 kb upstream control region of the AChR ε subunit gene and the reporter gene β-galactosidase was shown to produce synapse-specific β-Gal expression in transgenic mice, indicating that accumulation of ε subunit mRNA at synaptic sites is also due to transcriptional mechanisms (Sanes et al., 1991).

The molecular nature of the nerve-derived signals that induce synapse-specific transcription is still not known. Nevertheless, these studies with transgenic mice effectively posed two criteria that any candidate ligand need to fulfill in order to be accepted as the endogenous inductive signal. First, the ligand should be able to induce AChR δ or ε subunit gene transcription. Second, this ligand should be present in the synaptic basal lamina and be stable following muscle and nerve degeneration.

Many attempts have been made to identify the factors that induce AChR synthesis in cultured myotubes. Numerous molecules emerged as candidates from these studies (Markelonis, et al., 1982; Knaack et al., 1986; New and Mudge, 1986; Jessell et al., 1979). Among them, CGRP (New and Mudge, 1986) and ARIA (Jessell et al., 1979) attracted the most attention. CGRP increases AChR synthesis in cultured chick myotubes and is present in spinal motor neurons (New and Mudge, 1986; Fontaine et al., 1986). Its stimulatory effect, however, is small (50%) and is limited to the AChR α subunit (Fontaine et al., 1987). Moreover, despite its effect on AChR synthesis on chick

myotubes, CGRP has no effect on the level of AChR mRNAs in mouse myotubes (Martinou, et al., 1991).

ARIA (acetylcholine receptor-inducing activity) was originally purified from chicken brain on the basis of its ability to stimulate the rate of AChR incorporation in cultured chick myotubes (Usdin and Fischbach, 1986; Falls et al., 1990). It remains the most promising candidate since it mimics the endogenous factor in a number of respects. First, ARIA increases the rate of AChR incorporation up to 10-fold and upregulates the mRNA level for the AChR α subunit in cultured chick myotubes (Harris, et al., 1988, 1989). Moreover, ARIA elevates the mRNA level for AChR α, γ, δ and ε subunit in primary mouse myotubes by severalfold, with the most potent effect on the ε subunit gene (Martinou et al., 1991). In rodents, developmental regulation of ε expression is distinct from the other AChR subunits in that its appearance requires innervation and its mRNA is always confined to the synaptic nuclei (Brenner et al., 1990; Martinou et al., 1991). Therefore, it has been suggested that the appearance of the ε subunit is strictly dependent on a neurotrophic factor; this is consistent with ARIA's prominent effect on ε subunit expression in vitro.

ARIA cDNA was recently cloned from chick and was shown to be encoded by the same gene that encodes Neu differentiation factor (NDF) , glial growth factor (GGF) and heregulin (Falls et al., 1993; Wen et al, 1992; Marchionni et al, 1993; Holmes et al., 1992). To avoid confusion over the terminology, a new term, neuregulin, was coined to replace the above nomenclature (Marchionni et al, 1993). In situ hybridization and Northern blot analyses indicate that neuregulin is highly expressed in spinal motor neurons (Falls et al., 1993; Marchionni et al, 1993), consistent with the idea that it plays a role in inducing AChR synthesis in the synaptic region. However, it was not known whether neuregulins are transported to the motor nerve terminal and whether they are incorporated into the synaptic basal lamina. In this study we used antibodies to demonstrate that neuregulins are present in the synaptic basal lamina, and that, like the signal that stimulates synapse-specific expression of the δ and ε subunit genes, neuregulin is capable of activating AChR δ subunit gene transcription. In addition, the same cis-element region that confers synapse-specific transcription for the AChR δ subunit in transgenic mice is sufficient to mediate its response to neuregulin. Moreover, we show that receptors for neuregulin, erbB3 and erbB4, are concentrated in the synaptic region of the muscle fiber. These results suggest strongly that neuregulin is a signal that stimulates synapse-specific transcription of AChR genes.

MATERIALS AND METHODS

Neuregulin Antibodies

A thirteen amino acid peptide (amino acids 197 to 209 in Neu differentiation factor) (Wen et al., 1992), which is conserved in the EGF domain of different neuregulin isoforms but is not present in EGF domains of other proteins (Wen et al., 1992; Holmes et al., 1992), was coupled to a MAP carrier core (Research Genetics, Huntsville, AL), and rabbits were immunized with the conjugate to produce polyclonal antibodies.

Immunofluorescence

Muscle frozen sections were prepared as described (Ohlendieck et al., 1991). To stain for neuregulin, frozen sections (10 μm) of the rat sternomastoid muscle were incubated with anti-neuregulin sera (diluted 1/1500) over night at 4°C, followed by fluorescein-conjugated secondary antibodies and tetramethylrhodamine α-bungarotoxin (TMR-α-BGT).

To stain for erbB3, unfixed frozen sections of rat intercostal muscle were incubated with either a rabbit polyclonal antibody (Santa Cruz Biotechnologies, diluted 1:100) against the carboxyl terminal 17 amino acids of HER3 (human erbB3), or a rabbit polyclonal antibody (#3185, courtesy of Dr. Carraway) against a peptide in bovine erbB3, overnight at 4°C, followed by fluorescein-conjugated secondary antibodies and TMR-α-BGT.

To visualize the distribution of erbB4, unfixed or fixed frozen sections of mouse soleus muscle were reacted overnight at 4°C with three different rabbit antisera (#616, #618, #622) raised against three different peptide sequences near the carboxyl terminus of murine erbB4 (#616 is against a peptide from amino acid 1185 to 1238, #618 is against a peptide from amino acid 1109 to 1137, #622 is against a peptide from amino acid 1116 to 1182), followed by fluorescein-conjugated secondary antibodies and TMR-α-BGT.

hGH assay

Conditioned media from either untreated or neuregulin treated C2 myotubes was harvested, and the amount of hGH secreted into the media was quantitated using a radioimmunoassay, according to the manufacturer's instructions (Nichols Institute).

RNase protection assay

RNase protection assays were performed as described previously (Simon et al., 1992). Briefly, radiolabelled antisense RNA was produced by in vitro transcription and

was hybridized with total RNA in hybridization buffer overnight at 42°C. Non-hybridized RNA was digested with RNase (A and T1) for 1 hour at 30°C. Following proteinase K digestion and phenol extraction, the protected RNA was precipitated by ethanol and fractionated in 5% denaturing polyacrylamide gels. RNase protection assays were quantitated with a PhosphorImager (Molecular Dynamics).

Immunoprecipitation

To immunoprecipitate erbB2, cells were lysed in RIPA buffer (20 mM HEPES, pH 7,4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 1 mM orthovanadate, 2 mM leupeptin, 1 mM pepstatin, 1 mM pefabloc, 1 mM aprotinin), and the lysate was cleared by centrifugation and incubated with an erbB2 monoclonal antibody (c-neu Ab3, Oncogene Sciences) for one and half hours at 4°C. ErbB3 was immunoprecipitated in a similar way, except that we used rabbit antibodies against erbB3 (C-17, Santa Cruz Biotechnology). The immunoprecipitated proteins were captured on Protein A-Sepharose beads and subsequently eluted with SDS protein sample buffer.

Western blot

Immunoprecipitated proteins were eluted from Protein-A Sepharose beads by boiling in sample buffer, fractionated by SDS-PAGE and transferred to nitrocellulose. To visualize phosphotyrosine, the blotted nitrocellulose filters were incubated with a recombinant phosphotyrosine antibody RC20H (Transduction Laboratories) for 1 hour at room temperature. To detect erbB2 and erbB3, the nitrocellulose filters were probed with antisera against erbB2 (6-2987 or C-18, Santa Cruz Biotechnologies) and rabbit antibodies against erbB3 (C-17, Santa Cruz Biotechnology), respectively. Horseradish peroxidase (HRP) conjugated antibodies were used to detect bound erbB2 and erbB3 antibodies, which was visualized by enhanced chemiluminescence (ECL, Dupont).

DNase footprinting

Footprinting was performed as described previously (Thanos and Maniatis, 1992). The plasmid containing the regulatory region (-181/+25) of AChR δ subunit gene was digested with XbaI and HindIII and was end-labelled using Klenow polymerase. The end-labelled DNA fragment was gel purified. After brief incubation with recombinant transcription factors, p50, p65, and ATF2, the labelled DNA was digested with DNase I. The digested DNA was fractionated on a 6% polyacrylamide gel. The gel was dried and exposed to X-ray film overnight.

Gel shift assay

C2 myotube nuclear extracts were prepared as described previously (Simon and Burden, 1993). Gel shift assays were performed as previously described (Simon and Burden, 1993). NFkB and ATF2 oligos were end-labelled with γ ^{32}P-ATP (NEN) and T4 polynucleotide kinase (NEB). Unincorporated nucleotides were removed by a Biospin-3 column (Bio-Rad). Radiolabelled oligos were incubated with muscle cell extract under standard binding conditions for 30 min at room temperature prior to fractionation on a 5% 0.5X TBE polyacrylamide gel.

Expression of neuregulin in bacteria

To obtain neuregulin for subsequent experiments, I sought to express the protein in a bacterial expression system. Previously it has been shown that a bacterially derived heregulin fragment, corresponding to the EGF-like domain, is sufficient to induce erbB2 tyrosine phosphorylation (Holmes, et al., 1992). Since tyrosine phosphorylation of a receptor tyrosine kinase is the only known event that elicits subsequent biological responses, it is thought that this small EGF-domain peptide is sufficient to mediate the biological responses conferred by the entire protein. Therefore, I attempted to express this peptide in bacteria.

A potential problem arises at this point. The EGF-domain peptide contains multiple cysteines that form intramolecular disulfide bonds. By analogy with EGF, these bonds are likely to be essential for biological activity of the molecule. However, the intracellular compartment of bacteria is reducing in nature and thus prevents the formation of disulfide bonds. In order to express a biologically active neuregulin peptide in the cell, I took advantage of an *E. coli* mutant strain, *trxB::kan* (courtesy of Dr. Jon Beckwith), which is deficient in thioredoxin reductase and has enabled cytosolic expression of several proteins which contains disulfide bonds that are required for enzyme activity (Derman et al., 1993).

A cDNA encoding the neuregulin EGF peptide sequence was obtained by PCR amplification from MDA-MB-231 cells and was inserted into the pQE9 vector (Qiagen), which introduces a 6-histidine tag at the amino-terminus of the peptide to facilitate purification. The cDNA sequence was confirmed by sequencing. The plasmid was then transformed into *txB::kan* cells and induced with IPTG to produce the peptide (Derman et al., 1993). Subcellular fractionation and purification indicated that the cells produce the neuregulin peptide, but unfortunately, the majority (over 95%) of the protein is present in inclusion bodies, suggesting that most of the protein is incorrectly folded. Nevertheless, I

managed to purify the soluble cytosolic fraction with a Ni-NTA affinity column. In spite of the low yield, the purified protein is biologically active, since it induces tyrosine phosphorylation of erbBs in the MCF-7 breast cancer cell line.

Production of neuregulin in insect cells

Since the bacterial expression system does not produce sufficient quantities of neuregulin for subsequent studies, I explored other expression systems. Insect cells express and modify proteins in a manner similar to mammalian cells and were chosen to express neuregulin. To this end, the entire extracellular segment of human neuregulin (β1 isoform) cDNA was obtained by PCR amplification, and this fragment was inserted into an insect expression shuttle vector (AcGp43), which introduces a leader sequence (signal peptide) to the amino terminus of the protein. Insect cells were co-infected with both the shuttle plasmid and a helper virus. Independent plaques were isolated and a tyrosine phosphorylation assay was used to measure the production of neuregulin activity. The highest-expressing plaque was purified, amplified and used for all subsequent production of neuregulin.

RESULTS

Neuregulin stimulates AChR δ transcription in cultured myotubes

The AChR subunit genes, including the δ and ε subunit genes, are transcribed selectively in myofiber nuclei located near the synaptic site (Simon et al., 1992; Sanes et al., 1991). Studies in transgenic mice have shown that the *cis*-acting sequences for synapse-specific gene expression of the δ subunit gene are contained in 181 bp of 5' flanking DNA (Simon et al., 1992). To determine whether neuregulin activates transcription of the δ subunit gene, we treated C2 muscle cells that were stably transfected with gene fusions between the 5' flanking region of the AChR δ subunit gene and the hGH gene with recombinant neuregulin. Figure 1 shows that neuregulin causes a 2.5-fold increase in hGH secretion from C2 muscle cells stably transfected with an AChR δ(-1,823/+25)-hGH or an AChR δ(-181/+25)-hGH gene fusion. In contrast, neuregulin has no effect on the amount of hGH secreted from C2 muscle cells containing a metallothionein promoter-hGH gene fusion (Fig. 1). Thus, neuregulin stimulates transcription of the AChR δ subunit gene, and the neuregulin response element is contained in the same 181 bp that confers synapse-specific expression in transgenic mice.

Neuregulin is present at the neuromuscular synapse

Selective expression of AChR genes in the synaptic nuclei of muscle is thought to be activated by a signal provided locally at synaptic sites (Burden, 1993; Simon et al., 1992; Sanes et al., 1991; Klarsfeld et al., 1991). To determine whether neuregulins, which are expressed in motor neurons (Marchionni et al., 1993; Falls et al., 1993), are present at synaptic sites, we prepared antibodies against neuregulins and used these antibodies in immunocytochemical experiments. Multiple isoforms of neuregulin are generated by alternative splicing (Mudge, 1993), raising the possibility that different neuregulin isoforms may have different cellular and/or subcellular distributions. Therefore, we considered it important to raise antibodies that are not isoform-specific but would recognize most, if not all isoforms of neuregulin. In this regard, we prepared antibodies against a peptide sequence within the EGF domain of neuregulin that is conserved among the different neuregulin isoforms but not among other proteins containing EGF domains (Wen et al., 1992; Holmes et al., 1992). Figure 2 shows that the anti-peptide antibodies stain synaptic sites in frozen sections of skeletal muscle. Synaptic sites are also stained in unpermeabilized whole mounts of muscle, indicating that the EGF domain of synaptic neuregulin is exposed extracellularly. Less intense antibody staining is observed within motor axons of intramuscular nerves (Fig. 2a). Antibody staining is not observed with pre-immune sera (Fig. 2c) and is inhibited by pre-incubation of immune sera with the peptide (Fig. 2e). Thus, neuregulin is concentrated at synaptic sites.

Neuregulin persists at synaptic sites following degeneration of muscle and nerve terminals

The signal that activates synapse-specific gene expression remains at synaptic sites following denervation (Simon et al., 1992), and previous studies indicated that this signal is located in the synaptic basal lamina (Jo and Burden, 1992; Brenner et al., 1992; Goldman et al., 1991). Our immunocytochemical data demonstrate that neuregulin is localized to synaptic sites, but this data does not allow us to determine whether neuregulin is associated with the nerve terminal and/or with the synaptic basal lamina. To determine whether neuregulin, like the signal that activates synapse-specific gene expression, remains at synaptic sites following denervation, we stained both denervated and denervated, damaged skeletal muscle with antibodies to neuregulin. Figure 3 shows that neuregulin remains at synaptic sites following denervation. In contrast, components of the nerve terminal, including the synaptic vesicle protein SV2 and neurofilaments are phagocytized and removed from synaptic sites following denervation (Fig. 3c, e). Because our antibodies recognize the EGF domain of neuregulin and because this domain is sufficient for signaling (Holmes et al., 1992), neuregulins that are present at denervated

synaptic sites are likely to be functional. However, because the intensity of anti-neuregulin staining is greater at innervated synaptic sites than at denervated synaptic sites, neuregulin is also likely to be associated with nerve terminals. Figure 3 also shows that neuregulin remains at original synaptic sites following damage to nerve and muscle (Fig. 3g). The persistence of neuregulin at original synaptic sites in the absence of nerve and muscle is consistent with the idea that neuregulin is contained in the synaptic basal lamina and is a signal that activates synapse-specific gene expression.

ErbB3 and erbB4 are expressed in mouse and rat skeletal muscle

The receptors for neuregulin in muscle are not known. However, neuregulin receptors have been studied in some detail in human breast cancer cells. Although neuregulin was purified on the basis of its ability to induce erbB2 (neu) tyrosine phosphorylation, and was thus thought to be the ligand for erbB2 (Wen et al., 1992; Holmes et al., 1992), recent studies indicate that erbB2 does not bind neuregulin in the absence of erbB3 or erbB4 (Plowman et al., 1993). These studies showed that erbB3 and erbB4 are the true receptors for neuregulin (Plowman et al., 1993; Carraway et al., 1994). ErbB2, erbB3 and erbB4 share extensive homology and they all belong to the same EGFR family. Since erbB3 lacks tyrosine kinase activity, signaling through erbB3 requires the oligomerization of erbB3 and a receptor tyrosine kinase that has kinase activity (Carraway et al., 1994). In the case of erbB4, neuregulin can induce both homo-oligomerization and hetero-oligomerization of erbB4 with erbB2, suggesting that homo-oligomers of erbB4 or hetero-oligomers of erbB2 and erbB4 are capable of transducing the neuregulin signal.

Skeletal muscle is thought to express erbB4 (Plowman, 1993) but not erbB3 mRNA (Kraus, 1989); potential expression of erbB2 in skeletal muscle has not been studied. I used an RNase protection assay to measure the abundance of erbB2, erbB3 and erbB4 mRNA in C2, L6 and rat primary muscle cells, and in innervated and denervated mouse skeletal muscle. Figure 4 and 5 show that erbB2 and erbB3 are expressed in all sources examined. In both C2 and L6 cells, erbB3 expression is induced during muscle differentiation (Fig. 4 and 5). Low levels of erbB4 expression were detected in innervated and denervated mouse skeletal muscle and rat primary myotubes, but its expression is not detectable in either C2 myoblasts or myotubes and is very low in L6 myotubes. The absence of erbB4 expression in cultured C2 cells raised the possibility that the low level of erbB4 seen in skeletal muscle is owing to Schwann cells rather than muscle cells. To determine whether Schwann cells are the sole source of erbB4 expression in skeletal muscle tissue, I dissected mouse and rat diaphragm muscle into synapse-rich and synapse-free regions and measured erbB4 mRNA in both regions. As shown in figure 10, erbB4

mRNA expression is detectable in both synapse-free and synapse-rich regions. Since the synapse-free region of muscle lacks Schwann cells and other perisynaptic cells, this result suggests strongly that muscle cells express erbB4.

ErbB2 and erbB3 are the predominant erbBs in both L6 myotubes and rat primary myotubes. In L6 myotubes, erbB2 and erbB3 expression is ~1,000-fold grater than erbB4. Although erbB4 expression is higher in primary rat myotubes than in L6 myotubes, erbB2 and erbB3 expression is still 25 to 50-fold higher than erbB4. Thus, the predominant erbB response in C2 and L6 muscle cells is likely to be mediated by erbB2 and erbB3 and not erbB4.

Neuregulin stimulates tyrosine phosphorylation of erbB2 and erbB3 in cultured myotubes

The RNase protection assay showed that erbB2, erbB3 and erbB4 are expressed in skeletal muscle, but it did not show whether they are capable of signaling. To determine whether the erbBs in muscle are stimulated by neuregulin and might participate in signal transduction, I determined whether erbBs in cultured muscle cells respond to neuregulin. Since the earliest event in receptor tyrosine kinase activation is its autophosphorylation, I determined whether erbB2 and erbB3 are phosphorylated on tyrosine residues in response to neuregulin treatment. Differentiated C2 and L6 myotubes were treated with neuregulin for 5 min, erbB2 and erbB3 were immunoprecipitated from cell lysates and Western blots of immunoprecipitated proteins were probed with an antibody to phosphotyrosine. As shown in figure 6, both erbB2 and erbB3 from C2 and L6 myotubes showed an increased level of tyrosine phosphorylation after neuregulin treatment, indicating that erbB2 and erbB3 in muscle are properly assembled and capable of responding to neuregulin. Unfortunately, I was not able to extend the analysis to erbB4, since it is expressed at a very low level in L6 myotubes and is not detectable in C2 myotubes.

ErbB3 and erbB4 are concentrated at the vertebrate neuromuscular synapse

We reasoned that if neuregulin is a signal that activates synapse-specific gene transcription, its receptors might also be concentrated at the synapse. This notion prompted us to examine the localization of erbB2, erbB3 and erbB4 in rat and mouse skeletal muscle. We used rabbit polyclonal antibodies (C-17, Santa Cruz Biotechnologies) against the C-terminal 17 amino acids (1307-1323) of human erbB3 to stain rat muscle frozen sections. As shown in figure 7a, erbB3 staining is concentrated at adult synaptic sites. Pre-incubation of erbB3 antibody with its epitope peptide completely abolished the synaptic staining, demonstrating specificity for this peptide sequence. Another erbB3 antibody (#3185) raised against a peptide (amino acid 1048 to 1059) in

bovine erbB3 also stains synaptic sites of adult muscle. Taken together, these results support the idea that erbB3 is localized at synaptic sites in adult muscle.

We used three different erbB4 rabbit sea (#616, #618, and #622, courtesy of Dr. Cary Lai, Scripps Institute) raised against three different peptide sequences within the C-terminal region of mouse erbB4 to stain mouse muscle frozen sections. Each erbB4 antiserum selectively stains synaptic sites (Fig. 7c and table 2). To control for the specificity of staining, one of the erbB4 serum (#616) was incubated with its epitope peptide prior to staining procedures. Pre-incubation with the peptide substantially reduced the intensity of synapse staining, indicating that the peptide sequence is present in the molecule localized at synaptic sites.

Of the many erbB2 antibodies we used (see table 1), only one (6-2987), which is poorly characterized, stains synaptic sites. Three different antibodies (Ab3, Ab4 and anti-Her2 (Stein et al., 1994), courtesy of Dr. Margolis), which stain CHO cells expressing erbB2, fail to stain synaptic sites. Two antibodies, C-18 and K15 (Santa Cruz Biotechnologies), stain synaptic sites weakly. However, both antibodies stain intramuscular nerves more strongly and their synaptic staining is out of register with TMR-α-BGT staining, suggesting that the staining is associated with Schwann cells rather than postsynaptic muscle fibers. Although the 6-2987 antiserum reacts strongly with erbB2 but not erbB3 or erbB4 in Western blots, it may react with other antigens. Taken together, our data does not support the idea that erbB2 is concentrated at synaptic sites.

Since erbB2, erbB3 and erbB4 all belong to the same gene family and share extensive homology, there was legitimate concern as to the specificity of the peptide antibodies used. To determine whether the anti-erbB antibodies cross-react with each other, Western blots of the immunoprecipitated erbB2, erbB3 and erbB4 were probed with each anti-erbB antibody. Figure 9 demonstrates that each anti-erbB antibody recognizes only its own antigen and does not cross-react with the other erbBs. Therefore, our data indicate that erbB3 and erbB4 are concentrated at synaptic sites.

To determine whether erbB staining is associated with nerve terminals or the postsynaptic membrane, we stained denervated muscle with the antibodies. Within 48 hours after denervation, nerve terminals have degenerated and are phagocytized by perisynaptic cells, which effectively eliminates nerve terminal-associated antigens. Figure 8 shows that erbB3 and erbB4 staining remains at denervated synaptic sites, indicating that erbB3 and erbB4 are associated with the postsynaptic muscle. Therefore, not only the signal, neuregulin, but also its receptors, are concentrated at the synapse. This result further strengthens the idea that neuregulin is a signal that stimulates synapse-specific gene transcription.

Distribution of neuregulin, erbB3 and erbB4 during early stages of synapse formation

Synapse-specific transcription of the AChR δ subunit gene is detectable in new born mice (Simon et al., 1992), indicating that the nerve-derived signal which stimulates AChR δ subunit transcription acts early during synapse formation. We determined whether neuregulin is concentrated at synaptic sites at early stages of synaptogenesis. Frozen sections of skeletal muscle from rodents of different ages (1 day, 5 days, 8 days, 10 days and 14 days) were stained with the same neuregulin antibodies used in earlier studies. We were unable to detect neuregulin staining at synaptic sites until rodents reach 8 days of age (table 2).

We examined the distribution of erbB3 in muscle sections from rats of different ages (1 day, 5 days, 8 days, 10 days and 14 days), using the two anti-erbB3 antibodies (C-17, Santa Cruz Biotechnologies; and #3185, courtesy of Dr. Carraway) (table 2). Synaptic staining is weak and variable in neonatal rats, but becomes apparent 5 days after birth. The intensity of staining increases with age.

The distribution of erbB4 in young mice was determined by a panel of three peptide antibodies (#616, #618, #622), which stain synaptic sites in adult mouse muscle (table 2). Two of the three antibodies, #616 and #618, stain synaptic sites in neonatal mice. The intensity of staining is, however, weak compared with that in adult, but becomes strong 5 days after birth. The third antibody, #622, does not stain synaptic sites until mice reach 2 weeks of age.

Agrin induces aggregation of erbB3

We asked whether agrin is capable of clustering erbBs. To this end, we collaborated with Dr. Mendel Rimer in Dr. U.J. McMahan's laboratory. Dr. Rimer had previously shown that injection of plasmid DNA which directs expression of an active form of agrin into the nonsynaptic region of denervated rat skeletal muscle induces AChR clusters near the sites of injection. We stained denervated rat muscle injected with the agrin plasmid, with anti-erbB3 antibody (C-17, Santa Cruz Biotechnologies) and found that agrin induces erbB3 clusters near the injection sites, and in addition, erbB3 clusters colocalized precisely with AChR clusters. These results demonstrate that agrin can promote the formation of erbB3 clusters and raise the possibility that agrin may cause the localization of erbBs at synaptic sites.

By analogy with AChR and AChE, which are selectively transcribed by synaptic nuclei, we speculated that erbBs might be preferentially expressed by synaptic nuclei. Thus, we sought to determine whether erbB mRNAs are concentrated in the synaptic

region of the muscle. We dissected mouse and rat diaphragm muscles into synapse-rich and synapse-free regions, and measured erbB2, erbB3 and erbB4 mRNA levels in both regions. Figure 10 shows that erbB mRNA is expressed at comparable levels in synaptic and non-synaptic regions. AChR δ and ε subunit mRNAs were, however, more enriched in the synaptic region of mouse and rat diaphragm muscles respectively, indicating that our dissection does enrich for synaptic nuclei. Nevertheless, this crude assay does not allow us to conclude that erbB mRNAs are not enriched in the synaptic nuclei; since the dissected synapse-rich region still consists largely of non-synaptic nuclei, thus, a low level of erbB expression by non-synaptic nuclei would mask a potential accumulation of erbB mRNAs at synaptic sites. In situ hybridization using erbB RNA probes will be the most appropriate method to determine whether erbB mRNAs are concentrated at the synaptic sites.

Neuregulin does not affect the affinity of NFkB and ATF2 for the AChR δ control region

We do not know how the neuregulin signal propagates from the cell surface to the nucleus. We do know, however, that 181 bp of the AChR δ regulatory region is sufficient to confer the response to neuregulin. Therefore, at the final step of the signal transduction pathway, the signal has to impinge upon trans-acting factors that bind to this minimal control sequence. A database search indicates that this 181 bp region contains elements that may bind NFκB and ATF2 transcription factors. To determine whether the 181 bp control region is indeed capable of binding these transcription factors, we performed DNase footprint experiments with purified NFκB and ATF2 proteins. As shown in figure 11, NFκB and ATF 2 bind to the 181 bp control region, raising the possibility that neuregulin signaling might be mediated through these transcription factors.

One mechanism to increase the rate of transcription is to enhance the binding of stimulatory transcription factors to enhancers. I investigated the effect of neuregulin treatment on the affinity of NFkB and ATF2 for their cis-elements. C2 myotubes were exposed to neuregulin for 24 hours and nuclear extracts were prepared from either treated or untreated cells and were used in a gel retardation assay to determine whether binding had increased. As shown in figure 12, neuregulin treatment does not significantly affect the gel shift pattern of NFκB and ATF2 oligos. Therefore, our results does not support the idea that neuregulin signaling is mediated by altering the affinity of NFκB or ATF2 for the AChR δ subunit regulatory region.

DISCUSSION

Previous studies with transgenic mice defined two properties for a nerve-derived signal that activates synapse-specific AChR gene transcription. First, the signal should be capable of stimulating AChR δ subunit gene transcription. Second, the signal should be associated with the synaptic basal lamina. The present study demonstrates that neuregulin, a molecule previously shown to increase AChR subunit mRNA levels in cultured myotubes, is capable of activating AChR δ subunit gene transcription in a muscle cell line. Furthermore, the same 181 bp regulatory region from the AChR δ subunit gene that confers synapse-specific transcription is sufficient to mediate the response to neuregulin. Moreover, antibody staining shows that neuregulin is present in the synaptic basal lamina, since it remains at original synaptic sites following degeneration of nerve terminals and muscle fibers. Therefore, neuregulin fulfills both criteria anticipated from the studies in transgenic mice, and these results support the idea that neuregulin is the signal that activates synapse-specific gene transcription.

The finding that antibodies to neuregulin do not detectably stain synaptic sites in new-born rodents is not expected. Previous studies demonstrated that the AChR δ subunit gene is selectively transcribed by synaptic nuclei in neonatal mice (Simon et al., 1992), suggesting that the signal that is responsible for local transcription is present at synaptic sites by birth. Our failure to detect neuregulin staining at synapses in new-born mice seems to be inconsistent with the idea that neuregulin directs synapse-specific transcription at this stage. However, it is possible that neuregulin is present in synaptic sites in new-born mice, but its level is too low to be detected with our antibodies. Consistent with this notion, the level of AChR δ subunit gene expression in synaptic nuclei appears to be less in neonatal mice than in adult mice, raising the possibility that the level of AChR δ subunit expression is limited by the availability of neuregulin. Therefore, it remains possible that neuregulin is the signal that directs synapse-specific transcription during early stages of synapse formation.

Our staining results support the idea that erbB3 and erbB4 are localized at synaptic sites in adult rodents. We showed that two different anti-erbB3 antibodies stain synaptic sites in adult muscle. In addition, we showed that synaptic staining with one of the anti-erbB3 antibodies, C-17 (Santa Cruz Biotechnologies), is blocked by pre-absorption with the appropriate peptide and that this anti-erbB3 antibody does not cross-react with either erbB2 or erbB4. Therefore, our results strongly suggest that erbB3 is localized at synaptic sites. Several lines of evidence support the notion that erbB4 is present at synaptic sites. First, three different erbB4 antibodies (#616, #618, #622) raised against three different sequences in erbB4, stain synaptic sites. Second, synaptic staining by antibody #616 was blocked by pre-incubation of the antibody with its epitope peptide, but

not by an irrelevant peptide. Finally, antibody #616 does not cross-react with erbB2 or erbB3 on Western blots. Taken together, our results indicate that erbB3 and erbB4 are concentrated at synaptic sites in adult muscle.

We showed that erbB4 is localized at synaptic sites in new-born rodents. Two of the three anti-erbB4 antibodies, #616, #618, stain synaptic sites in new-born mice, while the third antibody, #622, does not stain synaptic sites until 2 weeks after birth. Synaptic staining with #616 in new-born mouse muscle is specific because the staining is blocked by absorption with its epitope peptide. The intensity of #616 and #618 staining in new-born mice, however, is weaker than in adult mice, indicating that the concentration of erbB3 and erbB4 at synapses increases during development. The lack of synaptic staining in new-born mice with #622 raises the possibility that multiple isoforms of erbB4 might be expressed and be differentially regulated during skeletal muscle development. It is possible that an isoform containing the peptide sequence recognized by #622 is not present at synapses early during development and appears two weeks after birth.

ErbB3 does not appear to be localized at synaptic sites at birth. Synaptic staining by C-17 anti-erbB3 antibody (Santa Cruz Biotechnologies) in neonatal rats is undetectable in most cases and is very weak when it is detected. Definitive synaptic staining with this antibody is observed with 5-day old rats. This result suggests that localization of erbB4 at synapses might precede that of erbB3.

We were not able to detect erbB2 at synaptic sites consistently. Although one antibody to erbB2, 6-2987, stains synaptic sites, all of the other anti-erbB2 antibodies we used fail to stain synaptic sites (see table 1). Control experiments showed that five anti-erbB2 antibodies (C-18, K15, Ab3, Ab4, anti-Her2) that fail to stain postsynaptic muscle membrane do stain CHO cells expressing erbB2. Since 6-2987 is an unpurified serum, it is possible that 6-2987 reacts with another antigen, which happens to be localized at synaptic sites. Nevertheless, 6-2987 is not likely to cross-react with erbB3 and erbB4, since we showed that 6-2987 does not react with erbB3 and erbB4 on Western blots (Fig. 8). The identity of this cross-reacting antigen at synapses is not clear. Our conclusion from these studies is that either erbB2 is not expressed at synaptic sites, or that its level of expression at synapses is too low to be detectable by the available anti-erbB2 antibodies.

Based on our results, three models can be proposed to explain signaling events that mediate synapse-specific transcription. According to the first model, two signals are required to produce synapse-specific transcription at all developmental stages. An unknown signal would act early during synapse formation to activate AChR α, β, γ and δ subunit transcription in synaptic nuclei. This signal would not stimulate transcription of the ε subunit. The level of this signal, however, would decline after birth, and be replaced

by another signal as synapse matures. The second signal might be neuregulin, which has a more potent effect on the ε subunit gene (7-fold) than on the γ subunit gene (2-fold). The replacement of the early signal by neuregulin could explain the developmental change in AChR composition, from $\alpha_2\beta\gamma\delta$ to $\alpha_2\beta\varepsilon\delta$. Transcription of the AChR α, β and δ subunit genes, in contrast, would be regulated similarly by both the early signal and neuregulin, and therefore, the rate of transcription of these genes would not be affected by the replacement of the early signal with neuregulin.

According to the second model, neuregulin is the only signal that activates synapse-specific transcription. Its differential effect on either AChR γ subunit or ε subunit would arise from different intensities of neuregulin signaling at different stages of synapse development. During early stages of synapse development, the level of neuregulin and its receptors, erbB3 and erbB4, is low; and this low level of neuregulin signaling might be sufficient to stimulate transcription of AChR α, β, γ and δ subunit genes, but not the ε subunit gene. As the levels of neuregulin and erbB3 and erbB4 increases with age, enhanced neuregulin signaling would act to repress transcription of the γ subunit gene while activating transcription of the ε subunit gene. Transcription of AChR α, β and δ genes at synaptic sites would increase as neuregulin signaling increases. Therefore, the response of the AChR subunit genes to neuregulin would be divided into three classes. The first class includes the α, β and δ subunits, which would respond to low and high levels of neuregulin signaling. The second class would be represented by the γ subunit, whose transcription would be activated by a low level of neuregulin signaling and repressed as neuregulin signaling increases. Transcription of the ε subunit gene, on the other hand, would require a high level of neuregulin signaling.

According to the third model, neuregulin would be the only signal that activates synapse-specific transcription. Different neuregulin receptors at different developmental stages would determine whether the γ or ε subunit gene would be activated. During early stages of development, erbB4 is more abundant than erbB3 at synapses, favoring homo-oligomerization of erbB4. In this model, activated erbB4 homo-oligomers would selectively activate transcription of the γ subunit gene and not the ε subunit gene. As synapse development progresses, the ratio of erbB3 to erbB4 at synapses would increase and favor the hetero-oligomerization of erbB3 and erbB4. Activated erbB3-erbB4 hetero-oligomers would preferentially stimulate transcription of the ε subunit gene while having no effect on γ subunit gene. Transcription of the AChR α, β and δ subunit genes, however, would be insensitive to the change in neuregulin receptors.

The definitive way to determine the role of neuregulin in synapse-specific transcription will be to eliminate neuregulin from motor neurons and study the effect of

loss of neuregulin on synapse-specific transcription. Gene targeting would be the method of choice to eliminate neuregulin. However, since neuregulin is expressed in the central nervous system and heart muscle, in addition to motor neurons, mice that lack neuregulin might not survive long enough to allow an examination of synapse-specific transcription. Thus, it might be necessary to selectively eliminate neuregulin expression in motor neurons by tissue-specific gene replacement.

Likewise, it will be necessary to eliminate erbB3 and erbB4 expression in muscle to determine their role in synapse-specific transcription. Like neuregulin, erbB3 and erbB4 are also expressed in other tissues such as heart and brain in addition to motor neurons. Therefore, selective elimination of erbB3 and erbB4 in muscle by tissue-specific gene replacement is likely to be necessary to generate viable mice.

Alternative splicing produces many different neuregulin isoforms (Mudge, 1993). It has been suggested that different isoforms may bind to different receptors and elicit different cellular responses. In support of this idea, recent experiments indicate that two isoforms, neuregulin α and β, exhibit different affinities for erbB3 and erbB4 (Lu et al., 1995b). Neuregulin β and its EGF-like domain peptide have an 8 to 10 fold higher affinity for erbB3 and erbB4 in NIH3T3 cells than neuregulin α or its EGF-like domain peptide. Moreover, neuregulin α and its EGF-like domain peptide show no mitogenic effect on NIH3T3 cells transfected with erbB3, and they have a weak effect on erbB4-transfected NIH3T3 cells. In contrast, neuregulin β and its EGF domain peptide show strong mitogenic effect on both erbB3 and erbB4 transfected cells. Since the EGF domain from neuregulin α and β differ only in the sequence between the fifth and sixth cysteine residue, this region is now thought to contribute to receptor binding and biological activity. We do not know which isoform is associated with the neuromuscular synapse, because the antibody we used is raised against a peptide sequence in the EGF-like domain that is identical between the α and β isoforms. Antibodies that distinguish between α and β isoforms should help to resolve this issue.

Most of the neuregulin isoforms identified so far contain a predicted transmembrane domain and lack amino-terminal signal sequences (Falls et al., 1993; Wen et al, 1992; Marchionni et al, 1993; Holmes et al., 1992). Only one isoform, GGFHBS5, which lacks a predicted transmembrane domain but contains a signal peptide, has been shown to encode a secreted factor when expressed in COS-7 cells (Marchionni et al., 1993). Our immunostaining results show, however, that this secreted isoform is not present at the neuromuscular synapse (data not shown). Therefore, it is quite possible that neuregulins present at the synapse are initially produced as membrane-associated isoforms. Since neuregulin staining persists following nerve and muscle damage, at least

some neuregulins are associated with the synaptic basal lamina, and these neuregulins most likely derive from the membrane-associated forms by proteolytic cleavage. Indeed, there is evidence that some neuregulin activity can be recovered in the conditioned medium when membrane bound forms are expressed in COS-7 cells (Falls et al., 1993; Wen et al, 1992). Moreover, recent expression studies demonstrate proteolytic processing of membrane-bound forms of rat and human neuregulins in COS-7 cells (Lu et al., 1995a). The same studies also identified preferred cleavage sites at the amino and carboxyl (juxtamembrane region) site of neuregulins, although they appear not to be identical to the natural cleavage sites found with originally purified rat NDF or human heregulin. Therefore, proteolytic processing of neuregulins might be cell-type specific. The proteases involved in neuregulin processing have not been identified. Some progress, however, has been made in identifying proteases that participate in proteolytic processing of EGF (Harano and Mizuno, 1994).

Neuregulins exhibit some affinity for heparin, raising the possibility that proteoglycans might be involved in neuregulin signaling. An interesting parallel is FGF, a growth factor that has an affinity for both proteoglycans and its cognate FGF receptor. In this case, proteoglycans have been shown to be a low-affinity receptor which aids in concentrating FGF and presenting FGF to the high-affinity FGF receptor. It is possible that a similar mechanism operates with neuregulins. In this context, it will be interesting to investigate whether neuregulins bind to proteoglycans, and whether such interaction can enhance its signaling capability.

At present, we do not know whether neuregulins are expressed in muscle. Previous studies by in situ hybridization do not demonstrate neuregulin expression in muscle (Marchionni, et al., 1993; Falls et al., 1993), although it is possible that the level of expression is too low to be detected by the technique. Agrin, the basal lamina-associated molecule that clusters AChRs, is expressed in both muscle and motor neurons. However, motor neurons and muscle express distinct isoforms of agrin, and it has been shown by nerve-muscle co-culture experiments that agrin derived from motor neurons, rather than from muscle, is responsible for aggregating AChRs (Reist et al, 1992). The function of muscle-derived agrin is not known, although it is found associated with induced AChR clusters in cultured myotubes (Lieth et al., 1991). In this context, it will be interesting to examine whether muscle might express neuregulin and whether a muscle-derived form is functional.

It is not known how erbBs become localized to the synaptic region of the muscle fiber. We have presented data that agrin, an extracellular matrix protein that clusters AChRs to the synaptic region, when injected into the non-synaptic region of the muscle

fiber, promotes the formation of erbB3 clusters. Therefore, agrin might be responsible, at least in part, for the localization of erbBs at synaptic sites. We do not know whether erbBs are locally transcribed and synthesized. Our preliminary studies do not support the idea that erbB mRNAs are concentrated in the synaptic region, when we measured their abundance in synapse-rich and synapse-free regions of muscle. However, our dissection is crude: 90% of the nuclei in the synapse-rich fraction are in fact non-synaptic nuclei, thus, even a low level of erbB expression in non-synaptic muscle nuclei would overwhelm a potential concentration effect of erbB mRNAs. A definitive answer has to await in situ hybridization analyses of erbB mRNAs.

There is evidence that the mRNA encoding AChE, 43k protein and N-CAM are also concentrated in the synaptic region of muscle (Moscoso et al., 1995). By analogy with AChR subunit gene, it is quite likely that these genes are also selectively transcribed by the synaptic msucle nuclei. In this respect, it will be interesting to determine whether neuregulin is capable of stimulating transcription of these genes. One approach will be to inject plasmids encoding secreted neuregulin in adult muscle and determine whether it is capable of inducing local transcription of these genes. Another approach, perhaps easier, is to determine whether neuregulin increases the steady state mRNA levels of these genes in cultured myotubes.

Little is know about how neuregulin activates AChR δ and ε subunit gene transcription. Studies in transgenic mice indicate that a proximal E-box in the AChR δ subunit control region is not required for synapse-specific gene transcription in vivo (Tang et al., 1994). Similarly, in cultured muscle cells, a reporter gene (CAT) driven by the ε subunit regulatory sequence with an mutated E-box responds similarly to neuregulin as the one driven by the wild type sequence, indicating that response to neuregulin can occur independently of the E-box and its associating myogenic factors (Chu et al., 1995). The same study also delineated 150 bp of ε regulatory region that is sufficient to confer neuregulin response. Unfortunately, there is little sequence homology between the AChR ε 150 bp regulatory region and AChR δ 181 bp regulatory sequence, suggesting that either synapse-specific transcription of these two genes is controlled by different transcription factors or the transcription factors recognize highly degenerate cis-elements. In any case, the ability to dissect the neuregulin-response element in cultured muscle cells should aid tremendously in the effort to identify the cis-elements conferring synapse-specific transcription.

It is not clear which intracellular signaling molecules are involved in propagating the neuregulin signal from the cell surface to the nucleus. Progress has been made, however, in identifying the signaling proteins that associate with erbB3 upon its auto-

phosphorylation (Prigent and Gullick, 1994). In this recent study, a chimera consisting of the extracellular domain of EGFR and intracellular sequence of erbB3 was stimulated with EGF, and the ability of intracellular sequences in erbB3 to bind other signaling molecules was assessed by co-immunoprecipitation. Both SHC and the p85 subunit of PI-3 kinase were shown to interact with erbB3 in an EGF-dependent manner. Phosphotyrosine peptides bearing potential phosphorylated tyrosines in the C-terminal tail of erbB3 were used to compete for binding to map the specific tyrosine that interact with p85 and SHC. Two phospho-tyrosines, at position Y1035, and Y1270 respectively, are potential sites for p85 binding, whereas one tyrosine, Y1309, is the putative residue that binds SHC (Prigent and Gullick, 1994). This study opens the door to search for additional SH2-domain containing molecules that interact with neuregulin receptors. It will be interesting to determine whether erbB3 in cultured myotubes also exhibit similar interactions with p85 and SHC.

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Table 4.1. Anti-erbB2 antibodies do not stain synaptic sites.

Antibody	Source	CHO cells staining	Synaptic staining
C-18	Santa Cruz Biotech	positive	weak (not in register with TMR-α-BGT staining); also stains intramuscular axons
K15	Santa Cruz Biotech	positive (weak)	as above
9G6	Santa Cruz Biotech	negative	negative
Ab1	Oncogene Science	negative	negative
Ab3	Oncogene Science	positive	negative
Ab4	Oncogene Science	positive	negative
anti-Her2	Dr. Margolis	positive (strong)	negative
6-2987	Dr. Schatzmann	nd	positive

The staining results with various anti-erbB2 antibodies on both CHO cells expressing erbB2 and muscle frozen sections are summarized. Both C-18 and K15 stain CHO cells expressing erbB2 but stain synaptic sites weakly. The synaptic staining by these two antibodies is in not precisely colocalized with TMR-α-BGT staining, suggesting that the staining is associated with Schwann cells rather than postsynaptic muscle membrane. Consistent with this idea, these two antibodies stain strongly Schwann cells associated with intramuscular motor axons. Three antibodies, Ab3, Ab4 and anti-Her2 (courtesy of Dr. Margolis), stain CHO cells but fail to stain synaptic sites. Ab1 and 9G6 do not stain either CHO cells or synaptic sites. 6-2987 stains synaptic sites, but we suspect that the staining is due to a cross-reacting antigen recognized by the antibody. nd, not determined.

Table 4.2. Synaptic staining of neuregulin, erbB3 and erbB4 during development.

	1 day	5 days	8 days	10 days	14 days	Adult
NRG	-	-	-/+	+	++	+++
erbB3	-	+	+	++	+++	+++
erbB4 (#616)	+	++	nd	+++	+++	++++
erbB4 (#618)	+	++	nd	+++	+++	+++
erbB4 (#622)	-	-	nd	nd	++	+++

-: no staining.
-/+: weak staining in 20% of the synaptic sites.
+, ++, +++, ++++: positive staining of increasing intensity.
nd: not determined.

Figure 4.1 Neuregulin activates AChR δ subunit gene transcription.
24 hour treatment of differentiated C2 muscle cells stably transfected with an AChR δ (-1823/+25)-hGH or an AChR δ (-181/+25)-hGH gene fusion with neuregulin results in over 2-fold increase in hGH expression. As a control, similar treatment of differentiated C2 muscle cells stably transfected with a metallothionein-hGH (MT-hGH) gene fusion with neuregulin does not significantly elevate hGH expression. Mean hGH secretion from two cultures treated with neuregulin and from four untreated cultures are shown. Mean increase in hGH expression from multiple experiments (n, number of repetitions for each experiment) is also indicated.

NEUREGULIN ACTIVATES TRANSCRIPTION OF THE ACHR DELTA SUBUNIT GENE

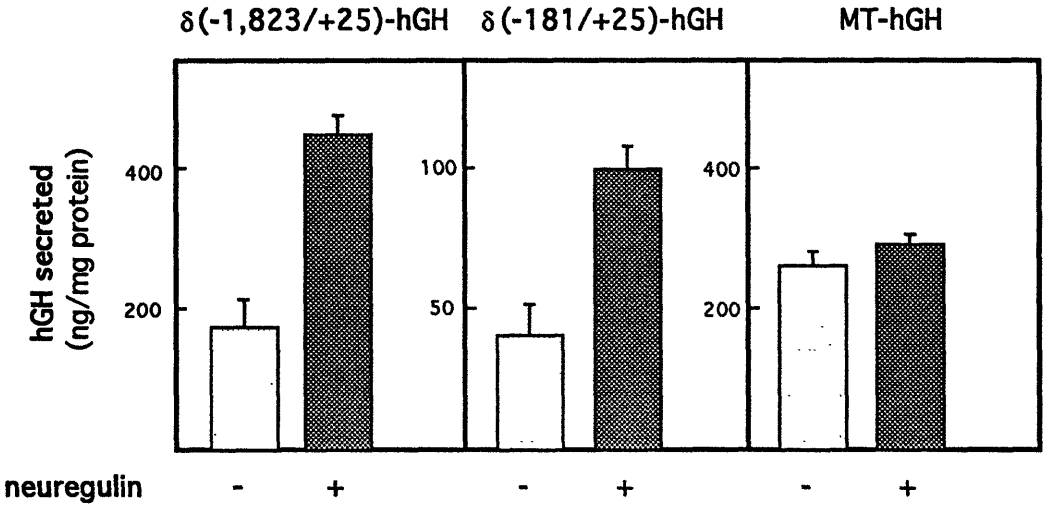


Figure 4.2. Neuregulin is concentrated at nerve-muscle synapses. Antibodies against neuregulin stain neuromuscular synapses (a. long arrows), which were identified by TMR-α-BGT (b, d, f). Less intense antibody staining is also detected within axons of intramuscular nerves (a, short arrows); the myelinating Schwann cells are not stained. Antibody staining is not observed with preimmune serum (c) and is inhibited by pre-incubation of the immune sera with the 13-amino-acid peptide (15 μm) (e). Scale bar, 15 μm.

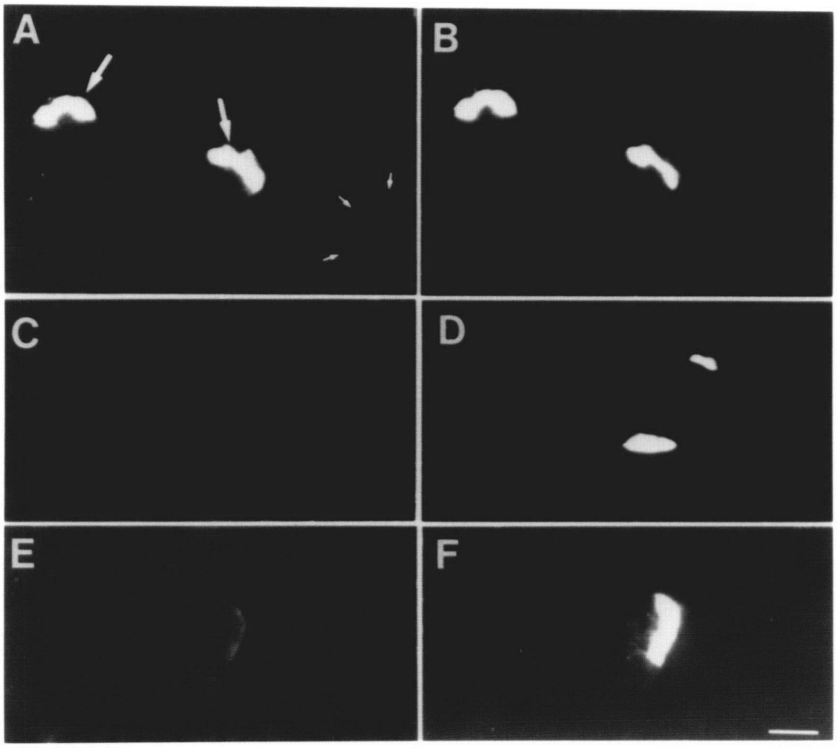

A
B
C
D
E
F

Figure 4.3. Neuregulin remains at synaptic sites in the absence of nerve and muscle.

Antibodies against neuregulin stain denervated neuromuscular synapses (a, arrows), which were identified by TMR-α-bungarotoxin (b). In contrast, antibodies against the synaptic vesicle protein, SV2, stain synaptic sites in innervated (c, arrow) but not in denervated (e) muscle. Antibodies against neuregulin stain original synaptic sites (g), identified by s-laminin staining (h), in the absence of nerve and muscle. A low level of synaptic staining, similar to that illustrated in (e), was observed in the absence of fluorescein-conjugated secondary antibodies and was due to TMR fluorescence detected in the fluorescein-selective channel. Scale bar, 18 μm.

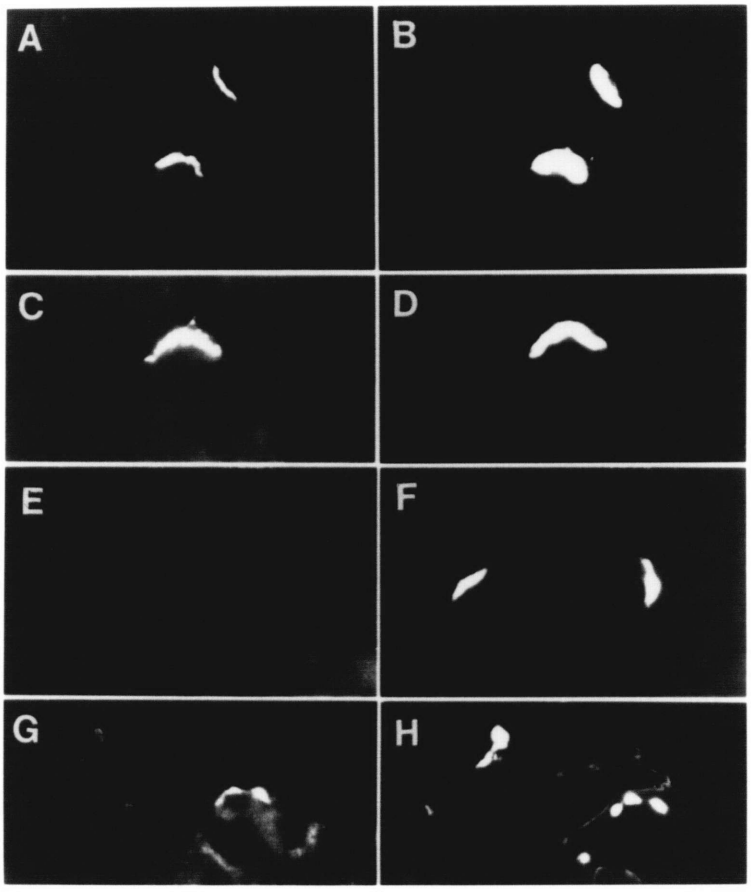

A
B
C
D
E
F
G
H

Figure 4.4. ErbB2, erbB3 and erbB4 are expressed in mouse skeletal muscle.

ErbB2 mRNA is expressed in innervated (INN) and denervated (DEN) adult mouse skeletal muscle (expression increases two to three-fold following denervation) and in C2 myoblasts (C2MB) and C2 myotubes (C2MT). ErbB3 mRNA is expressed in innervated and denervated mouse skeletal muscle (expression increases two to three-fold following denervation). Expression of erbB3 is activated during muscle differentiation, since erbB3 mRNA levels are low in C2 myoblasts and increase ~25-fold following myoblast fusion. The level of erbB3 mRNA in skeletal muscle is comparable to that in brain. ErbB4 mRNA is expressed at low levels in innervated and denervated adult mouse skeletal muscle. Expression of erbB4 mRNA is not detected in C2 myoblasts or myotubes. The level of erbB4 mRNA in skeletal muscle is low compared to that in brain.

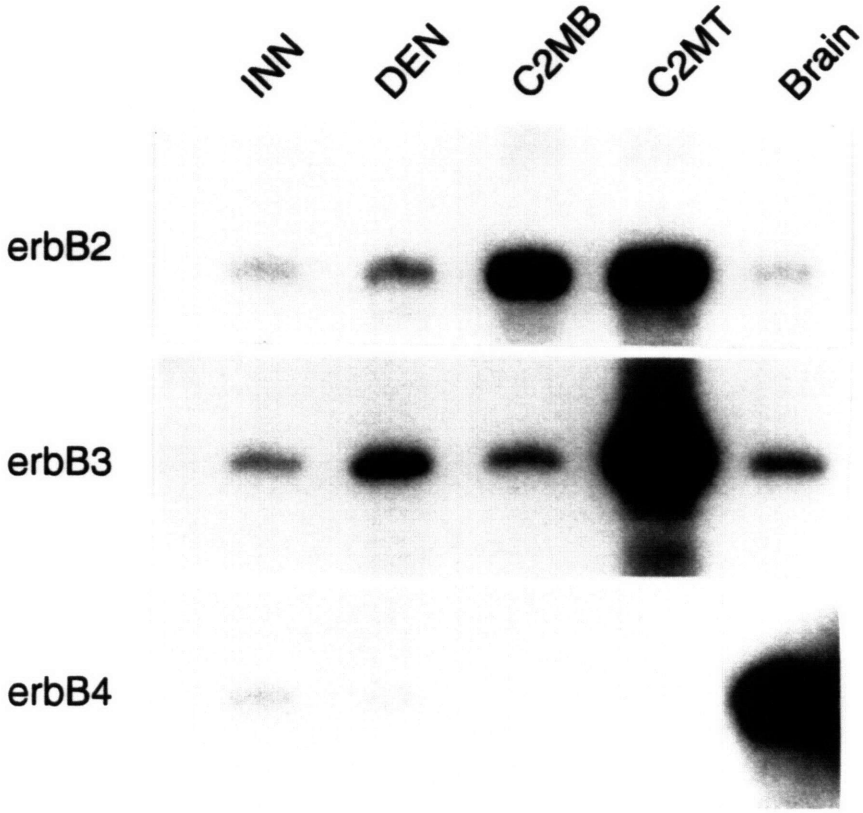

INN
DEN
C2MB
C2MT
Brain
erbB2
erbB3
erbB4

Figure 4.5. ErbB2, erbB3 and erbB4 are expressed in L6 and rat primary muscle cells.

ErbB2, ErbB3, ErbB4 mRNA levels in L6 myoblasts, L6 myotubes and rat primary myotubes were measured by RNase protection assay. ErbB2 is expressed in L6 myoblasts (L6 MB), L6 myotubes (L6MT) and rat primary myotubes. Expression of erbB2 is activated during muscle differentiation: the level of erbB2 mRNA in L6 cells increases approximately 6-fold after differentiation. ErbB3 is expressed in L6 myoblasts, L6 myotubes and rat primary myotubes. Expression of erbB3 is induced substantially during muscle differentiation: the level of erbB3 mRNA in L6 myotubes is approximately 60-fold more than that in L6 myoblasts. ErbB4 is expressed only in L6 myotubes and rat primary myotubes and not in L6 myoblasts. ErbB4 expression is also regulated by muscle differentiation, since its expression can only be detected in L6 myotubes but not in L6 myoblasts. The relative ratio of erbBs in L6 primary myotubes is: erbB2:erbB3:erbB4 = 650:1000:1, in rat primary myotubes, the ratio is: erbB2:erbB3:erbB4 = 27:50:1. The level of erbB4 mRNA in rat primary myotubes is ~10-fold higher than in L6 myotubes. ErbB2 and erbB3 expression, in contrast, is comparable in rat primary and L6 myotubes. ErbB2, erbB3 and erbB4 protection results presented here are exposed for different during of time.

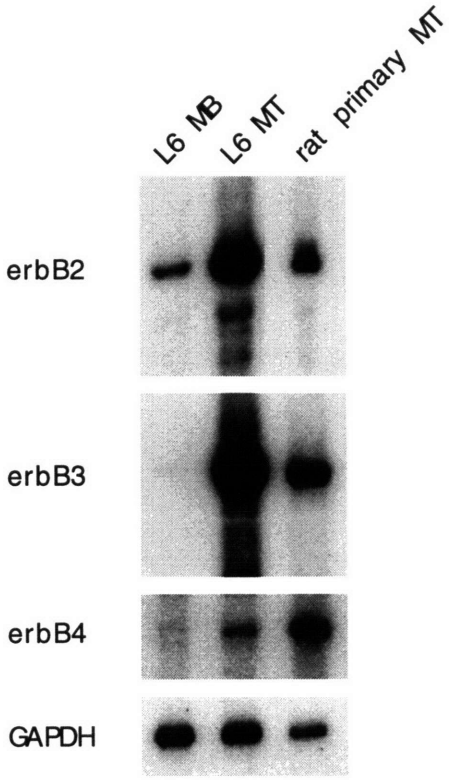
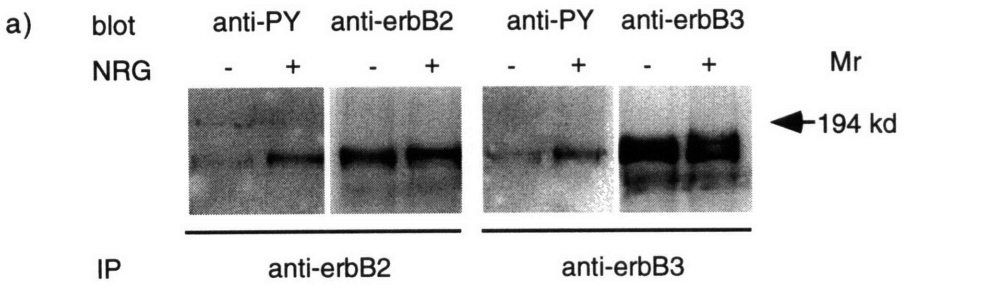

L6 MB
L6 MT
rat primary MT
erbB2
erbB3
erbB4
GAPDH

Figure 4.6. Neuregulin stimulates tyrosine phosphorylation of erbB2 and erbB3 in skeletal muscle cells.

Neuregulin (NRG) stimulates tyrosine phosphorylation of erbB2 and erbB3 in C2 (a) and L6 (b) myotubes. ErbB2 or erbB3 were immunoprecipitated with antibodies that are specific for each receptor from untreated C2 and L6 myotubes (-) or C2 and L6 myotubes treated with neuregulin (+). Phosphotyrosine incorporation was visualized with antibodies to phosphotyrosine (anti-PY), and erbB2 and erbB3 expression was visualized with antibodies (anti-erbB2 or anti-erbB3) that are specific for each receptor. ErbB2 and erbB3 migrate similarly in SDS-PAGE and have an apparent M_r of 180 kd.


a)
blot
anti-PY
anti-erbB2
anti-PY
anti-erbB3
NRG
- + - + - + - +
Mr
194 kd
IP
anti-erbB2
anti-erbB3

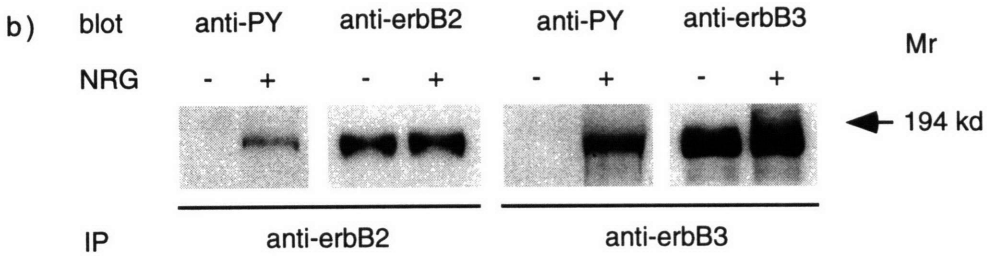

b)
blot
anti-PY
anti-erbB2
anti-PY
anti-erbB3
NRG
- + - + - + - +
Mr
194 kd
IP
anti-erbB2
anti-erbB3

Figure 4.7. ErbB3 and erbB4 are concentrated at nerve-muscle synapses. Frozen sections of skeletal muscle was stained with antibodies against erbB3 (C-17, Santa Cruz Biotechnologies) and erbB4 (#616). Both antibodies stain synaptic sites (A and C), as identified by TMR-α-BGT staining (B and D).

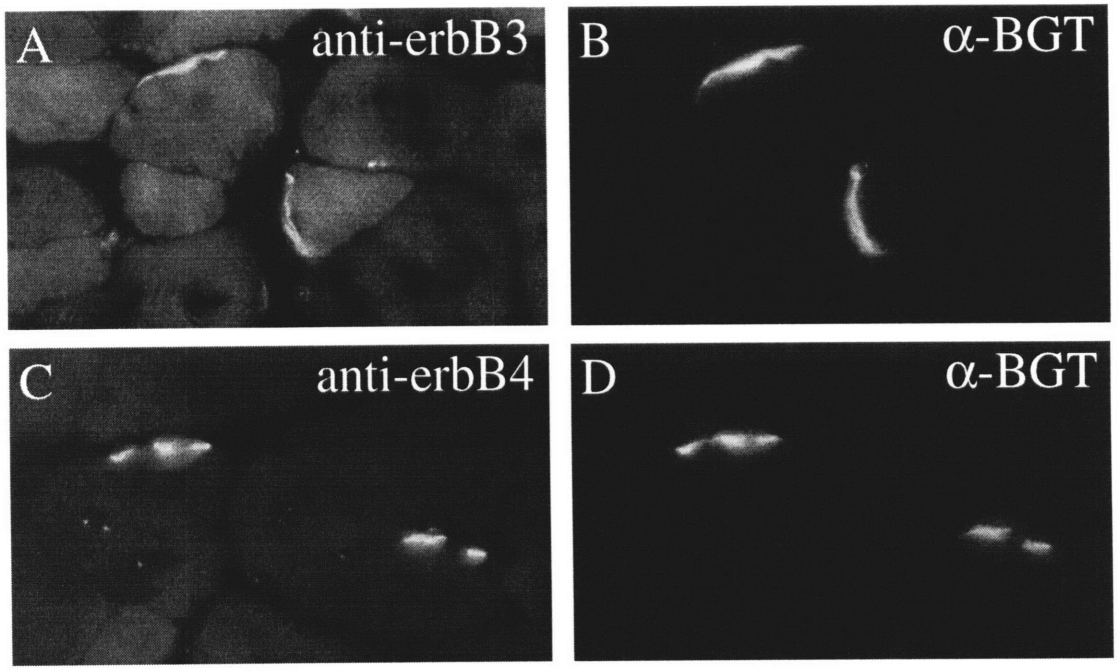

A
anti-erbB3
B
α-BGT
C
anti-erbB4
D
α-BGT

Figure 4.8. ErbB3 and erbB4 remain at synaptic sites in the absence of nerve terminal.

Frozen sections of 5-day denervated skeletal muscle were stained with antibodies against erbB3 (C-17, Santa Cruz Biotechnologies) and erbB4 (#616). Both antibodies stain synaptic sites (A and C), which are identified by TMR-α-BGT staining (B and D).

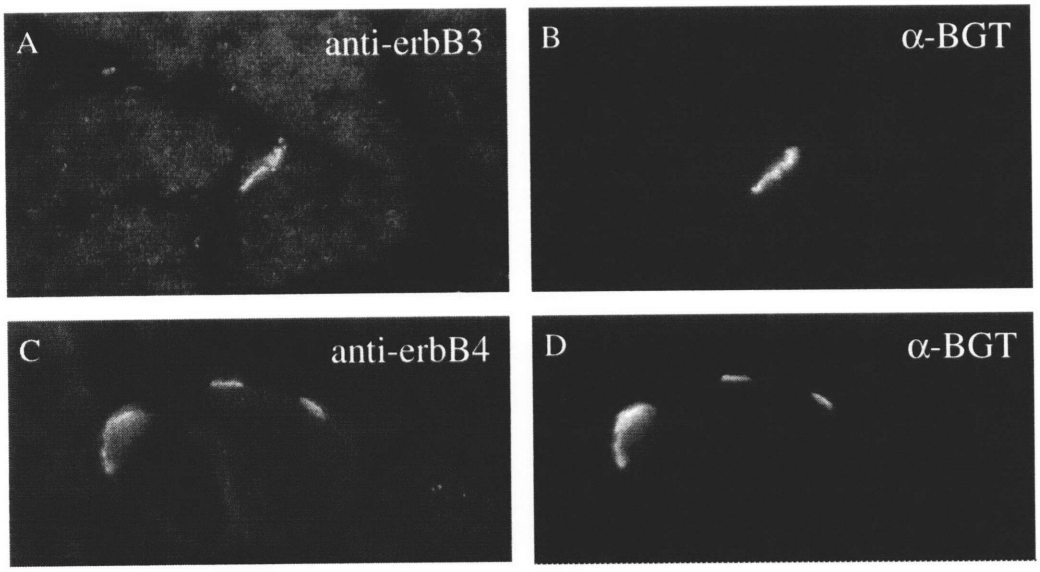

A
anti-erbB3
B
α-BGT
C
anti-erbB4
D
α-BGT

Figure 4.9. Antibodies to erbB2, erbB3 and erbB4 do not cross-react. ErbB2 and erbB3 were immunoprecipitated from C2 myotubes, erbB4 was immunoprecipitated from insect cells expressing mouse erbB4. Immunoprecipitated erbB2, erbB3 and erbB4 were blotted onto nitrocellulose filters and incubated with anti-erbB2 (6-2987), anti-erbB3 (C-17, Santa Cruz Biotechnologies) and anti-erbB4 (#616) separately. Each antibody only recognizes its own antigen and does not cross-react with other members of the same family.

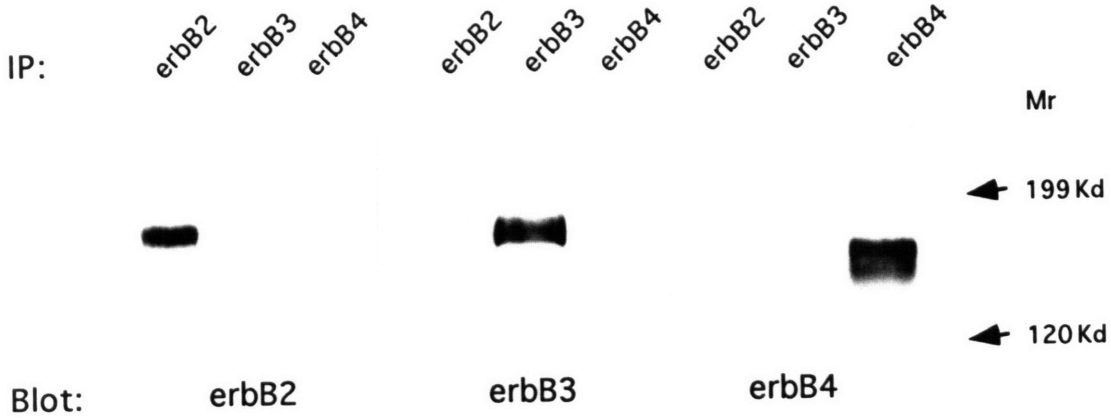

IP:
erbB2
erbB3
erbB4
erbB2
erbB3
erbB4
erbB2
erbB3
erbB4
Mr
199 Kd
120 Kd
Blot:
erbB2
erbB3
erbB4

Figure 4.10. ErbB2, erbB3 and erbB4 mRNAs are expressed in both synapse-rich and synapse-free regions of muscle.

Mouse (a) and rat (b) diaphragm muscles were dissected into synapse-rich (ep+) and synapse-free (ep-) regions, and mRNA levels for erbB2, erbB3 and erbB4 were measured by RNase protection assay. The levels of erbB2, erbB3 and erbB4 in synapse-rich and synapse-free regions are comparable. To normalize for the amount of RNA used for protection, GAPDH (rat) or actin (mouse) mRNA levels in synapse-rich and synapse-free regions were measured and were shown to be similar. In contrast, the level of AChR δ subunit mRNA in synapse-rich region of mouse diaphragm muscle is approximately 25-fold more than that in synapse-free region, the level of AChR ε subunit mRNA in synapse-rich region of rat diaphragm muscle is approximately 12-fold more than that in synapse-free region, demonstrating that synaptic-rich regions enrich for synaptic nuclei.

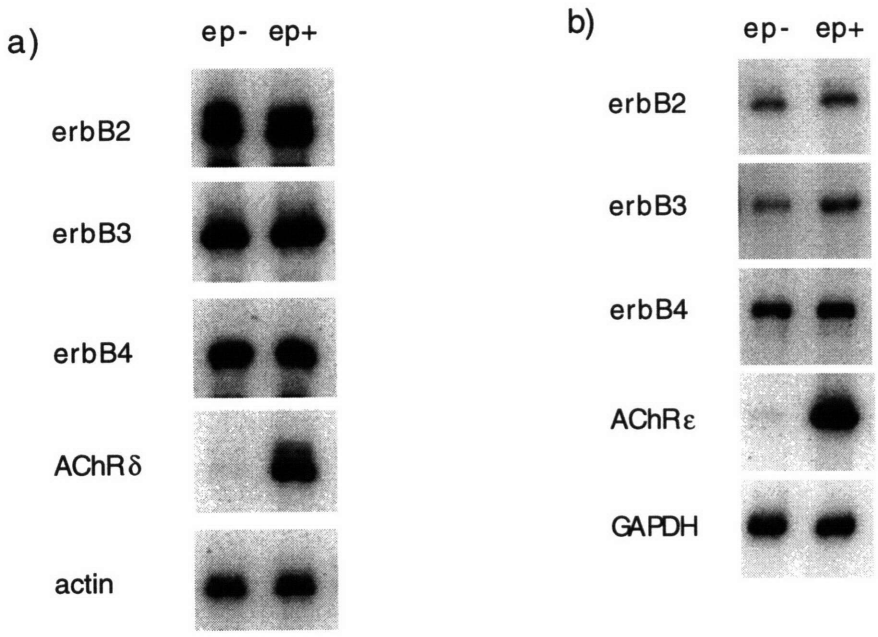
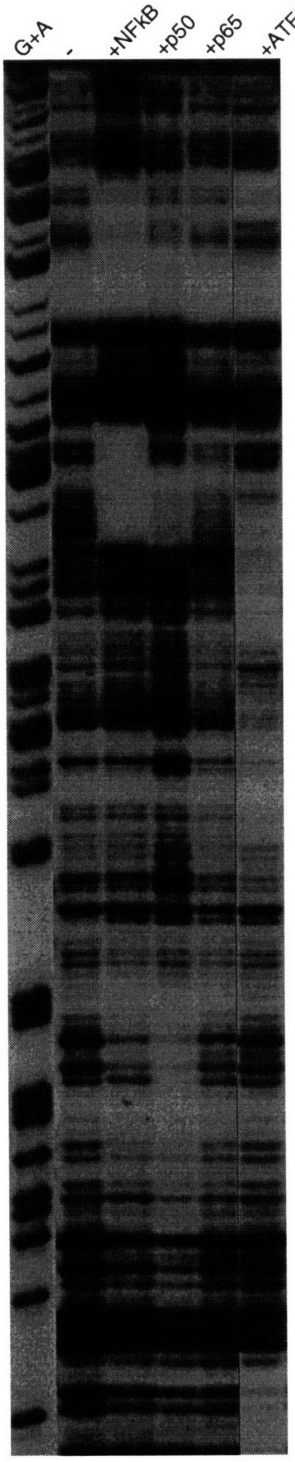

a)
ep-
ep+
erbB2
erbB3
erbB4
AChRδ
actin
b)
ep-
ep+
erbB2
erbB3
erbB4
AChRε
GAPDH

Figure 4.11. NFκB and ATF2 bind to 181 bp of AChR δ subunit regulatory region.

A DNA probe bearing the 181 bp of AChR δ subunit regulatory region was end-labelled. The probe was incubated with p50, p65, NFκB (p50+p65), or ATF2 recombinant proteins before being digested with DNase I (a). p50 and p65 alone does not protect the δ subunit regulatory region; together (as NFκB) protects two regions, -170/-138 and -111/-95 (b). ATF2 protects two nearly contiguous regions in the δ subunit regulatory region, identified as -95/-74 and -71/-53 (b).

a)

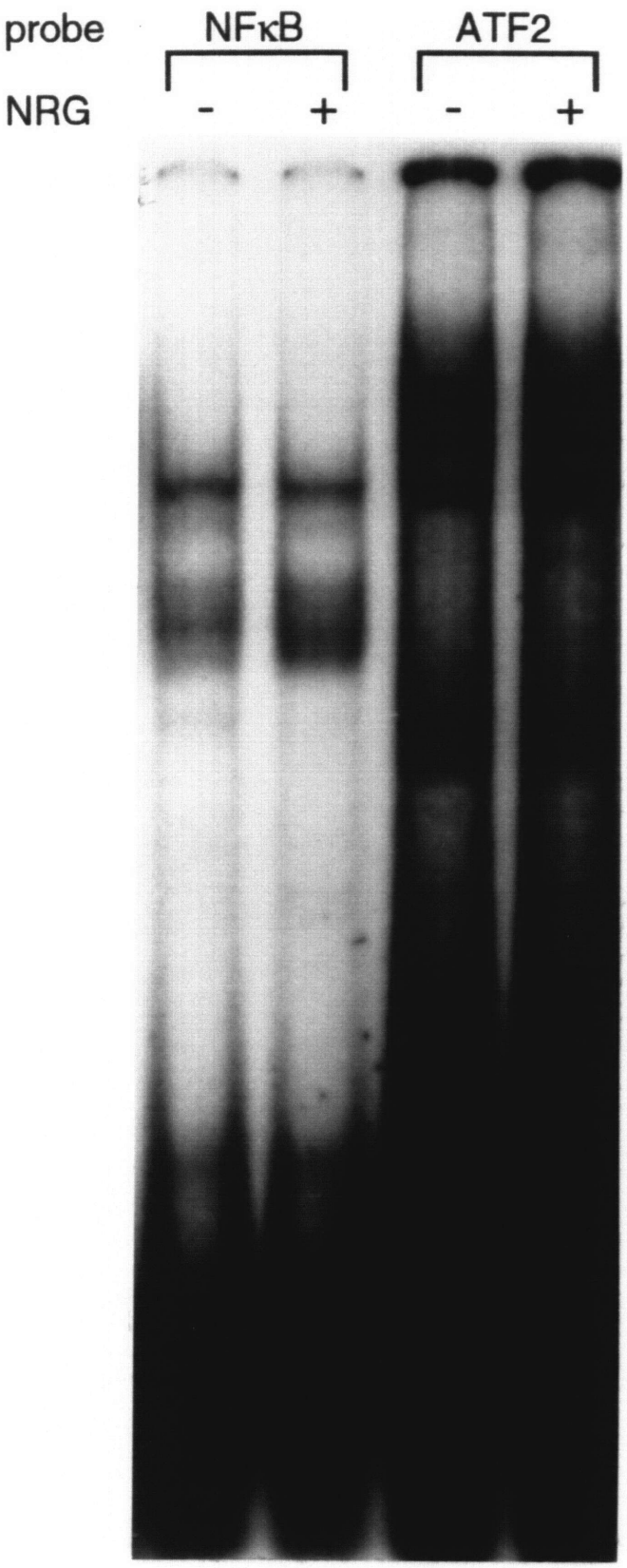


b)

NFκB
-181 -170 -160 -150
GGCCCCACCCTACCAACAATCTGTGACAATCCCTGCCTGG
NFκB
-140 -130 -120 -110
GATCTTTTCGTTCTGCCCTTGGCTCCTGCCCTAACTGGCAA
NFκB ATF2 ATF2
-100 -90 -80 -70
ACCCCACCCCCTCATCACCAGCTTTCAAGTATCAGATTGC
ATF2
-60 -50 -40 -30
GTTTCCGGCCTCTTCTTTCCAAACCCCTAAACCACCAGCA
-20 -10
CCTGTCCCCTTGCTTGCCTC

Figure 4.12. Neuregulin does not affect the affinity of NFκB and ATF2 for AChR δ subunit regulatory region.

Complementary oligos bearing the consensus binding sequence for NFκB and ATF2 were end-labelled with T4 polynucleotide kinase and annealed. The labelled oligos were incubated with nuclei extract prepared from either untreated (-) or neuregulin-treated (+) C2 muscle cells and were resolved on a 5% polyacrylamide gel. There is no difference in the binding pattern by either of the two oligos.


probe
NFκB
ATF2
NRG
-
+
-
+