Neuregulin-1 Induces Expression of Egr-1 and Activates Acetylcholine Receptor Transcription Through an Egr-1-binding Site

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Localization of acetylcholine receptors (AChRs) to neuromuscular synapses is mediated, in part, through selective transcription of AChR genes in myofiber synaptic nuclei. Neuregulin-1 (NRG-1) and its receptors, ErbBs, are concentrated at synaptic sites, and NRG-1 activates AChR synthesis in cultured muscle cells, suggesting that NRG-1-ErbB signaling functions to activate synapse-specific transcription. Previous studies have demonstrated that NRG-1-induced transcription is conferred by cis-acting elements located within 100 bp of 5' flanking DNA from the AChR epsilon subunit gene, and that it requires a GABP binding site within this region. To determine whether additional regulatory elements have a role in NRG-1 responsiveness, we used transcriptional reporter assays in a muscle cell line, and we identified an element that is required for NRG-1-induced transcription (neuregulin response element, NRE). Proteins from myotube extracts bind the NRE and NRG-1 treatment of the cells stimulates this binding. The ability of NRG-1 to stimulate formation of a protein–DNA complex with the NRE requires induction of protein expression. The complex contains early growth response-1 (Egr-1), a member of the Egr family of transcription factors, because proteins in the complex bind specifically to an Egr consensus site, and formation of the complex is inhibited by antibodies to Egr-1. NRG-1 induces expression of Egr-1 in myotubes, which presumably is responsible for the ability of NRG-1 to stimulate protein binding to the NRE. These results suggest that NRG-1 signaling in myotubes involves induction of Egr-1 expression, which in turn serves to activate transcription of the AChR epsilon subunit gene.

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Abbreviations used: AChR, acetylcholine receptor; MuSK, muscle-specific kinase; NRG-1, neuregulin-1; Egr-1, early growth response-1; hGH, human growth hormone; NRE, neuregulin-1 response element; EMSA, electrophoretic mobility shift assay; WT1, Wilms' tumor suppressor; ARIA, AChR inducing activity; ARE, ARIA response element; SRE, serum response element.

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Introduction

Shortly after motor axons contact developing myotubes, signals are exchanged between nerve and muscle that mediate the organization of a highly differentiated presynaptic nerve terminal and a highly specialized postsynaptic apparatus. Acetylcholine receptors (AChRs) are among the proteins that become localized to the postsynaptic region in muscle, and their localization to this small patch of the myofiber membrane during development is a hallmark of the inductive events of synapse formation.

It appears that two distinct signaling pathways mediate postsynaptic differentiation, including clustering of AChRs. In one pathway, agrin, a motor neuron-derived ligand, activates muscle-specific kinase (MuSK), a receptor tyrosine kinase, to stimulate the post-translational reorganization of proteins, including AChRs, in the muscle cell membrane. MuSK is also able to initiate some aspects of postsynaptic differentiation.
independently of agrin in muscle that is not innervated. A second pathway, whose signal is not known, leads to enhanced transcription of AChR genes in myofiber nuclei that are situated at synaptic sites.

Synapse-specific transcription has been demonstrated in transgenic mice that carry gene fusions between regulatory regions of AChR subunit genes and reporter genes. These transgenes are transcribed at a higher rate in myofiber nuclei near the synaptic site than in nuclei elsewhere in the myofiber, suggesting that motor neurons supply a signal to myofibers that activates AChR transcription in synaptic nuclei. Synapse-specific transcription leads to accumulation of AChR mRNA at synaptic sites, resulting in increased AChR protein synthesis in the synaptic region of the myofiber. RNAs encoding other synaptic proteins, including acetylcholine esterase, MuSK, Kapsyn, S-laminin, N-CAM, utrophin, and the regulatory subunit of protein kinase A, are also concentrated at synaptic sites, implying that synapse-specific transcription may be a general and important mechanism for clustering proteins at developing and adult neuromuscular synapses.

Neuregulin-1 (NRG-1), a widely expressed growth factor related to EGF, is currently the best candidate for the signal that activates synapse-specific transcription. NRG-1 is synthesized by motor neurons and concentrated at synaptic sites, like the signal that activates synapse-specific gene expression, NRG-1 is present in the synaptic basal lamina. The receptors for NRGs, ErbB3 and ErbB4, and the co-receptor ErbB2 are members of the EGF receptor family; both ErbB2 and ErbB4 are concentrated in the postsynaptic membrane at neuromuscular synapses. NRG-1 activates AChR gene expression in cultured muscle cells, and the same cis-acting region that confers NRG-1 responsiveness also confers synapse-specific transcription in transgenic mice. Further, a 5 bp regulatory element within this cis-acting region is required both for NRG-1-induced and synapse-specific transcription.

Mouse genetic approaches have been used to address whether NRG-1-mediated signaling is indeed required for synapse-specific gene expression. Mice lacking NRG-1, ErbB2 or ErbB4 are not informative because they die, owing to defects in cardiac development, several days prior to neuromuscular synapse formation. Mice lacking ErbB2 only in skeletal muscle are viable and have a mild deficiency in synaptic transmission and fewer AChRs at their neuromuscular synapses, providing further evidence that NRG-1-ErbB signaling may indeed have the suspected role in synapse formation. Because AChRs are not completely absent in these mice, ErbB3 and ErbB4 might partially compensate for loss of ErbB2. Mice that are heterozygous for an allele of NRG-1 that deletes the Ig-like domain (NRG1(+/-)) also have reduced AChRs and less efficient synaptic transmission. Motor neurons do not appear to be an essential source of NRG-1 for stimulating AChR transcription, however, because AChRs are expressed normally in mice lacking NRG-1 only in motor neurons. NRG-1 also is expressed by skeletal muscle cells, where it may function in an autocrine fashion due to the influence of the agrin-MuSK pathway, which is required for all aspects of neuromuscular synapse formation. In support of this idea, agrin can induce clustering of muscle-derived NRG-1 and ErbB receptors and stimulate AChR transcription through activation of ErbBs.

NRG-1-induced transcription is conferred by cis-acting elements located within 100 bp of 5' flanking DNA from the AChR ε subunit gene. Within this region, a consensus binding site for Ets proteins is required to respond to NRG-1, GABP, a heterodimer of GABPα, an Ets protein, and GABPβ, a non-Ets protein that enhances the DNA-binding activity of GABP, are the predominant proteins in myobule nuclear extracts that bind this element. NRG-1 stimulation does not increase the binding of GABP to DNA. NRG-1-stimulated phosphorylation of GABP causes changes to its quaternary structure and increases its transcriptional activity, suggesting that NRG-1 signaling stimulates transcription by increasing the transcriptional activity of GABP without affecting its DNA-binding activity.

Transcriptional reporter assays using P-19 teratocarcinoma cells, a non-muscle cell line, led to identification of a different element, a CA-rich sequence, that is required for NRG-1-induced transcription of the AChR ε subunit gene. NRG-1 stimulates binding of proteins from P-19 cells and from several other non-muscle cell lines to this element, and Sp1 is among the proteins that bind this element. The level of Sp1 in these cells is not altered by NRG-1 stimulation, indicating that Sp1 is most likely modified in these cells to increase its capacity to bind DNA. These studies did not evaluate the role of the CA-rich element in skeletal muscle cells, where expression of the AChR ε subunit gene is restricted. Because different cell types might exhibit different complements of transcriptional regulatory proteins that respond to NRG-1, it was not clear what function, if any, this element would have in muscle cells. We characterized the role of the CA-rich element of AChR ε subunit gene in cultured muscle cells and provide evidence for a different mechanism that could mediate a transcriptional response to NRG-1, one involving the transcription factor early growth response-1 (Egr-1). We show that the CA-rich element is required for NRG-1-induced transcription in muscle cells and that NRG-1 stimulates binding of Egr-1, rather than Sp1, to this element. Further, we show that expression of Egr-1 is increased by NRG-1, suggesting that NRG-1 signaling involves induction of Egr-1 expression, which in turn serves to activate AChR transcription.
Results

Identification of a neuregulin-1 response element in muscle cells

We used a transcriptional reporter assay to determine whether NRG-1-induced expression of the AChR ε subunit gene in skeletal muscle cells requires the CA-rich element. We generated AChR ε subunit (~228/+25)-human growth hormone (hGH) gene fusions using wild-type AChR gene regulatory sequences and using AChR sequences with various nucleotide substitution mutations within this element. We then stably transfected Sol8 muscle cells with these gene fusions and measured the amount of hGH expression from untreated or NRG-1-stimulated myotubes. Figure 1 demonstrates that the regulatory element being examined is required for NRG-1-induced expression in muscle cells. NRG-1 induces a 3.5-fold increase in hGH expression from myotubes transfected with a gene fusion containing the wild-type AChR regulatory region. In contrast, NRG-1 induces only a modest increase (1.4-fold, 1.5-fold) in hGH expression from myotubes transfected with any of the gene fusions containing mutations to the regulatory element. While these mutations reduce expression in response to NRG-1, they have little effect on the basal level of expression from untreated cells (Figure 1). Thus, the element being examined functions as a neuregulin-1 response element (NRE) in muscle cells.

NRG-1 stimulates protein binding to the NRE

We used an electrophoretic mobility shift assay (EMSA) to identify proteins in muscle cells that might interact with the NRE. We prepared whole cell extracts from myotubes that had been stimulated with NRG-1 for various times and evaluated protein binding to a labeled oligonucleotide probe containing the NRE. Figure 2 shows that proteins from myotube extracts bind the NRE and that NRG-1 treatment of the cells stimulates this binding. Maximal protein binding to the NRE occurs approximately one hour following NRG-1 stimulation and then returns to levels found in untreated cells within several hours (Figure 2(A)). Formation of a single protein-DNA complex is induced by NRG-1. Several faster migrating protein-DNA complexes were also detected in some experiments, but their appearance was inconsistent and their intensities varied (see also Figures 3–5). Further, NRG-1 treatment has no effect on the formation of these additional complexes. As a control for the quality and loading of cell extracts, we evaluated protein binding using an oligonucleotide probe containing an Ets site, which is known to bind proteins from myotube extracts independent of NRG-1-stimulation.28 Protein binding to the Ets site is equal from all the extracts (Figure 2(A)), confirming that NRG-1 specifically induces formation of a protein-DNA complex with the NRE.

We examined how the kinetics of AChR transcriptional induction by NRG-1 compares to the induction of protein binding to the NRE. To estimate when AChR transcription is initially activated during NRG-1 signaling, we evaluated reporter gene expression at various time points using cells transfected with AChR ε subunit-hGH gene fusions. Once NRG-1 signaling starts to induce AChR transcription in these cells, increased levels of hGH will begin to accumulate in the culture media. An increase in hGH levels in response to NRG-1 is apparent within 1.5 hours following NRG-1 stimulation (Figure 2(B)), indicating that by this time, NRG-1 has already begun to
To evaluate the specificity of NRG-1-induced protein binding to the NRE, we included excess unlabeled competitor oligonucleotides in the EMSA. We used wild-type NRE as a competitor and mutant versions containing various nucleotide substitutions, including those that we found to abolish NRG-1 responsiveness in muscle cells. Figure 3 shows that protein binding to the labeled NRE probe is specific, because an excess of wild-type NRE competes efficiently for binding, while an excess of any mutated NRE oligonucleotide either fails to compete or competes very poorly. Thus, the nucleotides that we found to be important for NRG-1-induced transcription in muscle cells are also required for protein binding to the NRE.

The ability of NRG-1 to stimulate binding of a particular protein(s) to the NRE could be the result of increased expression of this protein(s) in response to NRG-1. Alternatively, NRG-1 signaling could result in specific post-translation modifications that increase the DNA binding activity of this protein(s) without affecting its expression level. To determine if the ability of NRG-1 to stimulate protein binding to the NRE requires stimulation of protein expression, we used cycloheximide, an inhibitor of protein synthesis. We pre-treated myotubes with cycloheximide, stimulated them with NRG-1, and examined binding of cell extracts to the NRE. NRG-1 fails to stimulate protein binding from cells that have been pre-treated with cycloheximide (Figure 4), indicating that induction of expression of proteins is required for NRG-1 to stimulate formation of a complex with the NRE. This result suggests that NRG-1-mediated complex formation is due to increased expression of a NRE-binding protein(s) in response to NRG-1. Because NRG-1-induced AChR transcription also requires induction of protein expression, NRE-binding proteins may be among the proteins whose expression is required for inducing AChR transcription. Lack of complex formation with the NRE in cycloheximide-treated myotubes is not due to toxicity from cycloheximide.

Figure 2. NRG-1 stimulates protein binding to the NRE. (A) EMSAs were performed with whole cell extracts prepared from Sol8 myotubes (MT ex) stimulated with NRG-1. The radiolabeled oligonucleotide probe for the NRE extends from nucleotides −83 to −62 of the AChR ε subunit gene. The arrow indicates the position of the protein–DNA complex whose formation is stimulated by NRG-1. Maximal protein binding to the NRE occurs approximately one hour following NRG-1 stimulation and then returns to levels found in untreated cells within several hours. Protein binding to an Ets site probe detected in EMSAs is the same in all extracts, demonstrating equal loading of the samples. (B) Sol8 myotubes that had been stably transfected with AChR ε subunit-hGH gene fusions were re-fed with fresh culture media and stimulated with NRG-1. The amount of hGH secreted into the culture media from NRG-1-stimulated and untreated myotubes at various times is indicated. NRG-1 induces a statistically significant increase in hGH expression within 1.5 hours in myotubes transfected with an AChR ε (−228/+25)-hGH gene fusion (one-tailed t-test, p < 0.02), but not in myotubes transfected with a gene fusion containing a mutation (underlined) within the −76 to −66 region, indicating that AChR transcriptional activation by NRG-1 begins to occur during the time that protein binding to the NRE is induced.
because protein binding to an Ets site probe is not affected by cycloheximide treatment (Figure 4).

NRG-1-induced AChR transcription in muscle cells requires activation of the MAP kinase Erk.43–45 To determine if NRG-1-induced protein binding to the NRE is dependent on Erk, we performed EMSAs using extracts from NRG-1-stimulated myotubes that had been pretreated with PD98059, which blocks activation of Erk by inhibiting its upstream activating kinase MEK. NRG-1 fails to stimulate protein binding from cells that have been pretreated with PD98059 (Figure 4), indicating that activation of Erk is required for NRG-1-mediated complex formation. This result, taken together with the EMSAs using cycloheximide, suggests that Erk is involved in inducing expression of a protein(s) that binds the NRE in response to NRG-1.

Egr-1 binds the NRE

To identify proteins that might bind the NRE, we initially searched a database of previously characterized transcription factor consensus binding sites.46 This search revealed potential binding sites within the NRE for transcription factors from two different families, the Sp1/Kruppel-like family, which contains over 20 members with similar binding preferences,47 and the Egr family, which consists of Egr-1, Egr-2, Egr-3, Egr-4 and Wilms’ tumor suppressor (WT1).48,49 Numerous studies have shown that expression of Egr proteins can be induced by extracellular signaling molecules, and our EMSAs using cycloheximide suggest that expression of the protein(s) that binds the NRE is induced by NRG-1. Thus, Egr family members seemed like strong candidates for the protein(s) that binds the NRE following NRG-1 stimulation.

To determine if proteins that bind the NRE in response to NRG-1 are members of the Egr family, we included an excess of an unlabeled, competitor oligonucleotide containing an Egr consensus binding site in the EMSA. The Egr consensus oligonucleotide competes for binding at least as efficiently as a competitor containing the NRE (Figure 5(A)), indicating that NRE-binding proteins recognize the Egr consensus site. Binding to the Egr consensus site is specific because a mutated Egr oligonucleotide competes for binding very poorly. Further, an Sp1 consensus site fails to compete for binding. We also examined binding of myotube extracts to a labeled oligonucleotide probe containing the Egr consensus site. NRG-1 stimulates protein binding to the Egr consensus site, and this protein–DNA complex co-migrates with the one formed using the NRE probe (Figure 5(B)). Thus, a member(s) of the Egr family, or a protein(s) with a very similar binding preference, is among the protein(s) that bind the NRE in response to NRG-1.

We evaluated the protein–DNA complex with the NRE for the presence of particular Egr proteins. We incubated cell extracts from NRG-1-stimulated myotubes with antibodies to Egr proteins and determined whether these antibodies inhibit and/or super-shift the complex that forms in response to NRG-1. Antibodies that are specific for Egr-1 largely, if not entirely, inhibit the formation of the protein–DNA complex and super-shift the complex (Figure 5(C)). In contrast, antibodies that are selective for Egr-2 or Egr-3 and that can inhibit their ability to bind DNA fail to alter the protein–DNA complex. Using reverse transcription PCR, we found that the other members of the Egr family, Egr-4 and WT1, are not expressed in Sol8 myotubes (data not shown), so it seems unlikely that Egr-4 or WT1 would be present in the complex. Thus, Egr-1 appears to be the major, if not the only Egr protein present in the complex.
protein–DNA complex that forms in response to NRG-1. Because Egr-1 binds to the NRE early during NRG-1 signaling, we explored the possibility that other transcription factors might be interacting with the NRE during later times. We used EMSAs to examine protein binding to the NRE from extracts of cells that had been stimulated with NRG-1 for various times ranging from four to 24 hours. Protein binding to the NRE appears unchanged by these extended treatments with NRG-1, as compared to non-stimulated cells (Figure 5(D) and data not shown). Because Sp1 is known to bind the NRE in several types of non-muscle cells, we further examined whether the NRE-binding activity observed in extracts from NRG-1-stimulated myotubes contains Sp1. We incubated extracts from cells that had been stimulated with NRG-1 for various amounts of time with antibodies to Sp1, and found that these antibodies have no effect on protein binding to the NRE (Figure 5(D)). Thus, Sp1 does not appear to have a role in regulating transcription through the NRE in muscle cells.

Our EMSA results using cycloheximide suggest that expression of NRE-binding proteins is induced by NRG-1. Because Egr-1 binds the NRE, we examined whether expression of Egr-1 is responsive to NRG-1. Figure 6 shows that NRG-1 stimulates expression of Egr-1. Maximal expression of Egr-1 occurs approximately one hour following NRG-1 stimulation and then decreases to levels found in untreated cells within several hours. This is the same time-course by which NRG-1 stimulates formation of a protein–DNA complex with the NRE, suggesting that induction of Egr-1 expression is responsible for the ability of NRG-1 to stimulate protein binding to the NRE.

Discussion

Our results demonstrate that a binding-site for Egr proteins in the AChRδ subunit gene is required for transcriptional induction of the AChR gene by NRG-1. We show that Egr-1 binds this NRE, and that expression of Egr-1 is induced by NRG-1. These results suggest that NRG-1 signaling in muscle cells involves induction of Egr-1 expression, which in turn serves to activate AChR transcription.

Egr-1 expression is induced by many different stimuli, including various growth factors, conditions of cellular stress, and expression of oncogenes. These stimuli result in relatively rapid induction of Egr-1 expression, and Egr-1 can then regulate expression of target genes to mediate longer-term changes in gene expression. We show that Egr-1 expression in muscle cells is induced by NRG-1 and that Egr-1 binds the NRE of the AChRδ subunit gene, suggesting that the AChRδ subunit gene is a target for Egr-1 in NRG-1-stimulated cells. Previous studies identified a regulatory element termed AChR inducing activity (ARIA) response element (ARE) as being required for NRG-1-induced transcription of the AChRδ subunit gene in a muscle cell line. The sequences comprising the ARE partially overlap the NRE that we examined. Although studies with the ARE did not identify proteins that bind the element, mutations to the ARE that abolish NRG-1 responsiveness reside within the consensus binding site for Egr proteins, suggesting that the ARE identified in these studies is an Egr site. Sequences that are similar to the NRE are also found in regulatory regions of other synaptic genes, including the genes for the AChRε subunit and acetylcholine esterase, suggesting that Egr-1 might mediate the transcriptional response of these genes to NRG-1.

Increased expression of Egr-1 in response to
many different stimuli occurs at the transcriptional level. Presumably, NRG-1 also increases synthesis of Egr-1 in muscle by up-regulating its transcription, in which case components of the NRG-1 signaling pathway would interact with the transcriptional regulatory region of the Egr-1 gene. The regulatory sequences of the Egr-1 gene that mediate increased transcription in response to Egr-1 binds to the NRE. (A) EMSAs were performed with whole cell extracts from Sol8 myotubes stimulated with NRG-1 for one hour, a radiolabeled oligonucleotide containing the NRE, and unlabeled competitor DNA. The complex that forms in response to NRG-1 (arrow) is inhibited by an excess (−10- or 100-fold) of competitor DNA containing an Egr consensus site, and inhibition is at least as effective as with an NRE competitor. In contrast, unlabeled DNA containing a mutated Egr site competes for binding very poorly, and unlabeled DNA containing an Sp1 consensus site fails to compete for binding. (B) EMSAs were performed using extracts from Sol8 myotubes stimulated with NRG-1 for one hour and a radiolabeled oligonucleotide probe containing the Egr consensus site. NRG-1 stimulates formation of a protein–DNA complex with the Egr consensus site (arrow) that co-migrates with the one detected using the NRE probe. (C) EMSAs were performed using whole cell extracts from NRG-1-stimulated Sol8 myotubes and a radiolabeled oligonucleotide containing the NRE. Binding reactions were performed either in the absence of antibodies or after preincubation of cell extracts with the indicated antibodies. An antibody directed against Egr-1 inhibits the complex that forms in response to NRG-1 (large arrow) and causes the appearance of a super-shifted complex (small arrow). In contrast, antibodies to other Egr proteins, Egr-2 and Egr-3, and the unrelated protein Sp3, do not alter the protein–DNA complex. (D) EMSAs were performed using extracts from Sol8 myotubes stimulated with NRG-1 and a radiolabeled oligonucleotide containing the NRE. Treatment with NRG-1 for various times ranging from four to 24 hours does not alter protein binding to the NRE, as compared to non-stimulated cells (eight hour time point is shown, other time points not shown). Binding reactions were also performed after preincubation of cell extracts with antibodies to Sp1, and these antibodies do not alter protein binding to the NRE. As a control for antibody effectiveness, binding of myotube extracts to a radiolabeled oligonucleotide probe containing an Sp1 consensus site is inhibited by antibodies to Sp1 and Sp3, but not by irrelevant antibodies to the Flag epitope.
other stimuli are contained in the 5’ flanking region of the gene. This region includes multiple sites conforming to the serum response element (SRE), a regulatory element found in various early response genes, and mutational analyses revealed that the SREs are involved in the transcriptional response of Egr-1 to these stimuli.52–58

Signaling by many extracellular stimuli, including NRG-1, involves activation of the MAP kinase Erk, whose substrates include transcription factors that bind the SRE. Phosphorylation of these factors increases their ability to activate transcription of genes containing SREs.63 We show that NRG-1-stimulated formation of the protein–DNA complex that contains Egr-1 requires activation of Erk, suggesting that Erk is involved in inducing transcription of Egr-1 following NRG-1 treatment. Other studies demonstrate that the ability of NRG-1 to induce transcription of the AChR gene, a potential target of Egr-1, is dependent on activation of Erk.53–55 Taken together, these results are consistent with the idea that NRG-1 activates Egr-1 expression through Erk-mediated phosphorylation of factors that bind the SRE, and then Egr-1 binds the NRE to activate AChR transcription. In order to test this model, it will be important to determine whether NRG-1-induced transcription of Egr-1 is mediated by the SRE and its interacting proteins.

Egr proteins bind DNA via a zinc finger domain that is conserved between family members. The consensus binding site for these proteins is C-G-C-C-C/C-T-A/C-C-G-C.64 Although the Egr site in the NRE (CACCCCCC) differs somewhat from the consensus sequence, the NRE is able to bind Egr-1 that is present in cell extracts from NRG-1-stimulated myotubes. Using reverse transcriptase PCR, we found that Egr-2 and Egr-3, in addition to Egr-1, are expressed in myotubes that have been stimulated with NRG-1 (data not shown), yet Egr-2 and Egr-3 are not detected in the protein–DNA complex with the NRE in EMSAs. This is interesting that Egr-1 appears to be the major, if not only Egr protein present in the protein–DNA complex that forms in response to NRG-1. In principle, the predominance of Egr-1 in this complex could be owing to a greater abundance of Egr-1 than other Egr proteins in NRG-1-stimulated muscle. Alternatively, Egr-1 may bind the NRE with greater affinity than other Egr proteins. Although site selection experiments revealed that Egr-1, Egr-2, and Egr-3 have similar preferred binding sites,64 comparative studies of their binding to the NRE may reveal whether the NRE binds Egr-1 with the highest affinity.

We studied the function of the NRE in muscle cells, while previous studies used various other cell types. In several non-muscle cell lines, NRG-1 stimulates formation of a protein–DNA complex with the NRE that contains Sp1.31 The NRE contains overlapping consensus binding sites for Sp1/Kruppel-like proteins and for Egr proteins, so in principle multiple protein–DNA complexes could form with proteins from both families. We demonstrate that in muscle cells, NRG-1 stimulates formation of a protein–DNA complex that contains Egr-1. Further, Sp1 is not present in any protein–DNA complex that we detect by EMSA, because both an Sp1 consensus site competitor and an Sp1 antibody fail to interfere with complex formation (Figure 5). These differences in the composition of the protein–DNA complex in muscle cells and in certain non-muscle cell lines could be due to cell type differences in the relative abundance of potential NRE-binding proteins or of certain components of the NRG-1 pathway that act upon them.

_Cis_-acting elements involved in synapse-specific transcription have been analyzed by injection of AChR-LacZ gene fusions into muscles. One study used 5’ flanking sequences of the AChR α subunit gene, with nucleotide substitutions spanning throughout the region. This study found that mutations to the Ets site diminish synapse-specific transcription, while all other mutations, including to the NRE, fail to show any significant effect.25 These results suggest that the NRE is dispensable for synapse-specific transcription. Because the NRE is required for NRG-1-induced AChR transcription, signals in addition to or instead of NRG-1 could be involved in activating synaptic transcription in _in vivo_ or certain downstream components of the NRG-1 pathway might differ between muscle cell lines in culture and muscle cells _in vivo_. Alternatively, the lack of an effect of a particular mutation using the DNA injection approach may reflect limits on the sensitivity of this technique due to a relatively high level of non-specific background synaptic expression and the small number of fibers that take up and express injected DNA. The role of the NRE might be best studied by generating transgenic mice that contain AChR sequences mutated at the NRE.
If NRG-1 is actually involved in synapse-specific transcription, our results showing that NRG-1-induced AChR transcription requires a site that binds Egr-1 suggest a possible role for Egr-1 at synapses in vivo. Mice deficient in Egr-1 are viable, and no defects in synapses have been reported, making it unlikely that Egr-1 is required for activating AChR transcription at synapses. Egr-1 could have a redundant role in regulating synapse-specific transcription, so that if Egr-1 is missing, other Egr proteins are able to functionally compensate for its loss. Future studies will address the functional importance of particular Egr proteins in NRG-1-induced and synapse-specific transcription.

Materials and Methods

Cell culture

Sol8 myoblasts were grown on dishes coated with Matrigel (BD Biosciences) and fed with growth media (Dulbecco’s modification of Eagle’s medium (DMEM), 10% (v/v) fetal bovine serum, 0.5% (v/v) chick embryo extract, 50 μg/ml gentamycin). To induce differentiation into myotubes, cells were grown to confluency and the media was replaced with differentiation media (DMEM, 5% horse serum, 50 μg/ml gentamycin).

To prepare whole cell extracts, myotubes grown on 60 mm dishes that had been in differentiation media for three days were serum-starved for four hours and stimulated with NRG-1 (HRGβ1, R & D systems, 125 ng/ml) for various amounts of time. For experiments with inhibitors, 10 μg/ml cycloheximide or 100 μM PD98059 (Sigma) was added 30 minutes prior to NRG-1 stimulation and remained throughout NRG-1 treatment. Myotubes were lysed in 180 μl HNTG buffer (1% Triton X-100, 150 mM NaCl, 20 mM Hepes (pH 7.5), 10% (v/v) glycerol, 1.5 mM MgCl2) containing 1% (v/v) protease inhibitor cocktail (Sigma), 10 mM NaF, 30 mM Na2PO4, 1 mM Na2VO4. Extracts were briefly centrifuged to remove insoluble material. Protein concentration was measured by a Bradford assay (Bio-Rad).

Transcriptional reporter assay

An AChRε subunit (~228/+25)-hGH gene fusion was produced by inserting a PCR fragment, which was generated from rat genomic DNA, into the plasmid p0GH (Nichols Institute Diagnostics). Mutations in AChR gene sequences were introduced by PCR-based mutagenesis.66 Sol8 myoblasts in growth media were cotransfected with an AChRε subunit-hGH gene fusion and pSV2neo and selected with 750 μg/ml G418. Pools of stably transfected cells grown on 35 mm dishes were induced to differentiate using differentiation media. After 24 hours, cells were re-fed with differentiation media containing 1% horse serum and NRG-1 (HRGβ1, R & D systems, 125 ng/ml). Cells were re-fed with the same medium 24 hours later. Following an additional 24 hours, the medium was removed, and the amount of hGH secreted into the medium was measured by a radioimmunoassay (Nichols Institute Diagnostics). To analyze the kinetics of HGH expression, transfected myotubes that had been in differentiation media for three days were re-fed with DMEM containing NRG-1, and samples of media were removed at various times.

EMSA

Klenow was used to incorporate [α-32P]dCTP into double-stranded oligonucleotides that had one to three nucleotide overhangs at each end, and radiolabeled probes were purified using Bio-Gel P-6 columns (Bio-Rad). The sequences of radiolabeled and unlabeled competitor oligonucleotides for the NRE are nucleotides −83 to −62 of the rat AChR ε subunit gene (GCCTCTCCA CCCCACACCCAG), for the consensus Egr site are GTAA CGCCCCCGCAAGACTG, and for the consensus Sp1 site are GTCGCCGGCCCCGGATC GAAT. Binding reactions contained 12 μg protein from Sol8 myoblast whole cell extracts, 25 mM Hepes (pH 7.5), 50 mM KCl, 4 mM MgCl2, 5% glycerol, 0.1% NP-40, 0.3 mM DTT, 1 mg/ml BSA, 75 μg/ml poly[d(I-C)], and 30,000 cpm radiolabeled probe in a total volume of 20 μl. Some reactions also contained unlabeled competitor DNA. Reactions were initially set up that contained all components except probe and placed at room temperature for ten minutes. Probe was then added and the reactions were placed for an additional 15 minutes at room temperature and placed on ice for five minutes. For binding reactions containing antibodies, 1 μg of antibodies to Egr-1 (Santa Cruz Biotechnology, sc-189), Egr-2 (Covance, PRB-236P), Egr-3 (Santa Cruz Biotechnology, sc-191), Sp1 (Santa Cruz Biotechnology, sc-59), or Sp3 (Santa Cruz Biotechnology, sc-644) were incubated with whole cell extracts for one hour on ice prior to addition of other components. Complexes were resolved by electrophoresis (1.5 hours at 150 V) in a 5% (v/v) polyacrylamide gel (0.5 × Trisborate–EDTA), and gels were dried and exposed to X-ray film.

Western blotting

Proteins from myotube whole cell extracts were resolved by SDS-PAGE and transferred to PVDF membranes. Western blots were blocked with buffered non-fat dried milk and probed with an antibody to Egr-1 (Santa Cruz Biotechnology, sc-189) or Sp1 (Santa Cruz Biotechnology, sc-59). Antibody binding was detected by probing with peroxidase-conjugated anti-rabbit IgG secondary antibody (Jackson Immuno-Research) followed by chemiluminescence.

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