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Targeting Transcription to the Neuromuscular Synapse

Review

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Summary

Concomitant with innervation, genes coding for components of the neuromuscular junction become exclusively expressed in subsynaptic nuclei. A six-base pair element, the N box, can confer synapse-specific transcription to the acetylcholine nicotinic receptor δ and ε subunit, utrophin, and acetylcholine esterase genes. N box-dependent synaptic expression is stimulated by the nerve-derived signal agrin and the trophic factor neuregulin, which triggers the MAPK and JNK signaling pathways, to ultimately allow activation by the N box binding Ets transcription factor GABP.

The neuromuscular junction (NMJ) in vertebrates is a favorable model system to investigate the molecular mechanisms of synapse formation and plasticity. The acetylcholine nicotinic receptor (AChR) is a transmembrane ligand-gated ion channel composed of four subunits α, β, γ, and δ assembled into an heteropentamer [$\alpha_2\beta\gamma\delta$]. Concomitant with innervation, the AChR becomes highly concentrated at the level of the postsynaptic membrane. After birth, an ε subunit replaces the γ subunit to form the adult type of AChR, which possesses different ion channel properties. Many other molecules are also concentrated at the endplate. Among those present are the basal lamina proteins neuregulins, agrin, and acetylcholine esterase, the membrane receptors for agrin and neuregulins and the cytoskeletal associated proteins 43 K/rapsyn and utrophin (for review, see Sanes and Lichtman, 1999). Under this highly specialized supramacromolecular assembly lie a few specialized nuclei referred to as "fundamental nuclei" characterized by a clear chromatin and hypertrophied nucleoli.

The AChR is an excellent marker of synaptic differentiation. At early stages of development, *AChR* genes are expressed all along the embryonic myotube, whereas soon after a motor nerve terminal has contacted the myotube, *AChR* genes become exclusively expressed by the fundamental nuclei (for references to papers demonstrating compartmentalized transcription, see Duclert and Changeux, 1995, and Sanes and Lichtman, 1999). It is of considerable interest to understand the molecular mechanisms that control the normal development of this highly compartmentalized gene expression in vivo, in particular the precise role played by neural factors in the control of synapse-specific gene expression.

Two distinct mechanisms contribute to the regulation of *AChR* gene transcription. On one hand, nerve-evoked electrical activity in the muscle fiber represses transcription of *AChR* genes in the extra junctional area. On the other hand, trophic factors such as the acetylcholine receptor-inducing activity (ARIA/heregulin β/neu-differentiation factor/neuregulin β1) and also, indirectly, agrin (for review, see Sanes and Lichtman, 1999, and references therein) selectively enhance transcription of *AChR* genes in the postsynaptic domain. This review will primarily focus on this later aspect of transcriptional regulation at the neuromuscular synapse.

Identification of the N Box

A model proposed about 15 years ago (Changeux et al., 1987) for the compartmentalized transcription of *AChR* genes postulates a correspondence between synapse-specific signals and defined promoter elements (and transcription factors) within synapse-specific genes, together with parallel elements (and transcription factors) responding to electrical activity in extrajunctional areas. To identify the promoter elements involved in the targeting of transcription to the junctional domain, an in vivo direct DNA injection technique was used. Systematic mutagenesis of the *AChR* δ (Koike et al., 1995) and ε (Duclert et al., 1996) subunit promoters resulted in the identification of a single six-base pair element named the N box, required for synapse-specific expression of the reporter gene. In addition, when fused to a minimal promoter, the N box specifically increased synaptic expression of a reporter gene. Yet, the extent of the expression specificity was lower than in the context of the *AChR* δ or ε promoters, suggesting the existence of cooperativity with other promoter sites (Koike et al., 1995; see below).

The importance of the N box has been confirmed in transgenic mice where mutations in the N box of the *AChR* δ promoter completely abolished the expression of a reporter gene in adult innervated muscle. In these mice, expression of the transgene in denervated muscle was not affected, suggesting that the N box is necessary for synaptic expression but is not involved in the repression of synaptic genes expression by nerve-evoked electrical activity (Fromm and Burden, 1998).

A direct demonstration of the physiological importance of the N box in vivo was brought about by the discovery that a point mutation in the N box of the *AChR* ε subunit promoter causes congenital myasthenia (Nichols et al., 1999; Ohno et al., 1999). These congenital myasthenic patients carry mutations in the *AChR* ε promoter that precisely correspond to the experimental mu-

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tations known to abolish synaptic targeting of the *AChR* ε subunit gene in mouse (Schaeffer et al., 1998; Nichols et al., 1999). AChR expression was examined in one of these patients, and muscle biopsies were found to contain reduced levels of ε subunit mRNA (Nichols et al., 1999).

The control of transcription by the N box is not limited to the *AChR* δ and ε subunit genes. The *utrophin* gene promoter contains an N box that is essential for the synaptic expression of utrophin (Gramolini et al., 1999; Khurana et al., 1999). Moreover, the acetylcholine esterase gene promoter contains four N boxes. One among these, located in the first intron, is crucial for the synaptic expression of acetylcholine esterase (Chan et al., 1999). The N box is thus a critical element in targeting the transcription of several major synaptic genes to the motor endplate.

The N Box Recruits the Ets Class Transcription Factor GABP

Adult mouse muscle, as well as cultured myotubes, contains a factor (or factors) that specifically interacts with the N box sequence. One factor was purified from cultured muscle cells on a DNA affinity column and comprises a heterodimer composed of 58 and 43 kDa polypeptides corresponding to the Ets-related transcription factor GABP (Schaeffer et al., 1998; Khurana et al., 1999). This finding is consistent with the fact that the N box contains the GGAA sequence characteristic of the binding sites for the transcription factors belonging to the Ets family. The 58 kDa GABP α subunit binds DNA through its Ets domain. The 43 kDa GABP β subunit does not contact DNA but contains ankyrin repeats that mediate its interaction with GABP α, thus strengthening the interaction of the α subunit with DNA (Batchelor et al., 1998).

GABP β is encoded by two closely related, alternatively spliced genes, *GABP β1* and *GABP β2*. Each of these genes yields two alternatively spliced products. *GABP β2* expression could not be detected in muscle or cultured myotubes, and the relative level of expression of the alternatively spliced forms of GABP β1 in muscle has not been investigated (Schaeffer et al., 1998; Fromm and Burden, 1998). The binding of the GABP α/β1 complex to the N box results in transcriptional activation from the *AChR* δ and ε promoters. Indeed, antisense oligonucleotides directed toward GABP β1 or dominant-negative mutants of GABP α or β block N box-mediated transcriptional activation from the *AChR* δ and ε promoters in cultured myotubes (Schaeffer et al., 1998).

Briguet and Ruegg (2000) have shown that a GABP β dominant-negative mutant that blocks activation by the N box in cultured myotubes also blocks agrin-induced postsynaptic differentiation in vivo (also see the further section on agrin). In addition, expression and aggregation of synaptic proteins encoded by the *AChR* ε gene, and by the N box containing genes *utrophin* and acetylcholine esterase, is significantly reduced at the ectopic synapses. This provides a direct evidence that in vivo, as in cultured myotubes, GABP mediates the activation of synaptic genes. The involvement of Ets transcription factors in synapse formation is not unprecedented. Indeed, the inactivation of the Ets factor *ER81* by homologous recombination abolishes the formation of functional connections between a group of muscle sensory neurons and motor neurons (Arber et al., 2000).

Although GABP α expression is stronger in subsynaptic nuclei, *GABP* subunit genes mRNAs are present in both the synaptic and extrasynaptic muscle compartments (Schaeffer et al., 1998). At least four alternative (or complementary) mechanisms may restrict activation by GABP to the postsynaptic domain in response to a synaptic "neurotrophic" signal. First, since the shortest spliced form of GABP β does not contain the putative transcriptional activation domain, differential expression of two GABP splice variants could restrict the transcriptionally active forms of GABP to subsynaptic nuclei. This possibility could be tested by examining the distribution and subcellular localisation of the GABP α and β polypeptides in muscle. A second plausible mechanism could be that in the extra synaptic area one or both GABP subunits are sequestered into the cytoplasm and/or are degraded more rapidly than in the synaptic zone. A third mechanism could involve an Ets factor with repressor activity such as ERF that binds the N box in extra-synaptic nuclei instead of GABP. However, no N box binding activity other than GABP is detectable in muscle extracts, although more than 99% of the nuclei they originate from are extra-synaptic. Alternatively, GABP itself could act as a repressor in the extra synaptic nuclei. Such a dual activator/repressor function has indeed already been shown for GABP (Genuario and Perry, 1996).

A fourth mechanism that could be invoked is the specific upregulation of the GABP transactivation potential in subsynaptic nuclei by the signaling pathways triggered by a synaptic trophic factor. Two such factors, capable of stimulating AChR expression, were respectively described several years ago: CGRP (calcitonin gene related peptide) (Fontaine et al., 1986) and ARIA (acetylcholine receptor inducing activity) (Falls et al., 1993). CGRP is present in the motor nerve ending and enhances *AChR* genes expression in cultured myotubes (Fontaine et al., 1986). Yet, knockout mice for α*CGRP* do not show major changes in the morphology of the NMJ (Lu et al., 1999; Salmon et al., 1999). The closely related βCGRP, which is produced by a different gene, might compensate for the loss of αCGRP. However, the endplates from α*CGRP* knockout mice did not reveal significant CGRP-like immunoreactivity. ARIA was initially purified from chick brain on the basis of its ability to stimulate AChR expression in cultured myotubes (Falls et al., 1993). ARIA is also known as heregulin β, neu-differentiation factor, or neuregulin β and encodes a 42 kDa protein belonging to the neuregulin 1 family of EGF-like polypeptides, a group of 14 proteins that are generated from the alternative splicing of a single gene (see Burden, 1998). Genetic inactivation of a subset of neuregulin isoforms (the Ig-like domain containing form) is lethal before any neuromuscular junction is formed. However, analysis of heterozygous mutants revealed that these animals exhibit signs of congenital myasthenia and show a 50% decrease in the RNA coding for AChRs (Sandrock et al., 1997). In addition, in vivo muscle injection of a neuregulin expression vector stimulates the expression of a reporter gene controlled by the syn-

apse specific *utrophin* promoter (Gramolini et al., 1999). These results support the idea that, in vivo, neuregulins contribute to *AChR* genes upregulation at the NMJ.

Neuregulins and Their Receptors

Since the N box is the key promoter element of several synapse-specific genes, and since neuregulin is the best candidate factor to elicit synapse-specific transcription, it is anticipated that the N box and GABP are the downstream targets of the signaling pathways triggered by neuregulins. In addition, Ets transcription factors are classical targets of the MAP kinase signaling pathway through which neuregulins mediate their action. Finally, gene expression regulation by neuregulins via Ets transcription factors is not unprecedented. In breast cancer cells, upregulation of the Ets factor PEA3 transcriptional activity by neuregulins, via the ERK and JNK signaling pathways, has indeed been reported (O'Hagan and Hassell, 1998).

Neuregulins receptors belong to the same family as the EGF receptor: the erbB family of tyrosine kinase receptors. Neuregulins can bind to various dimers of erbB subunits (erbB2/erbB3, erbB2/erbB4, erbB3/erbB4, and erbB4/erbB4) and cause their phosphorylation. In innervated skeletal muscle, erbB2, erbB3, and erbB4 are clustered at the NMJ (Altiok et al., 1995; reviewed in Burden, 1998). The distribution of erbB subunits at the rat NMJ has recently been reexamined more accurately using confocal microscopy, and it appears that if both erbB2 and erbB4 are localized in the muscle postsynaptic membrane, erbB3 is only detectable in presynaptic terminal Schwann cells (Trinidad et al., 2000). The functional muscle receptor for neuregulins in rat thus seems to be either the erbB2/erbB4 heterodimer, or an erbB4 homodimer, or both. The localization of erbB3 in the presynaptic rather than postsynaptic compartment facilitates the interpretation of two intriguing observations made by Merlie and colleagues (Moscoso et al., 1995). First, preferential synaptic expression of synaptic genes in mouse embryos takes place before erbB3 is detectable at the NMJ. Second, in *rapsyn*-deficient mice, synaptic transcription is not affected, although erbB3 is not concentrated anymore at the NMJ. In both cases, terminal Schwann cells appear to be absent—in the first case because synaptic transcription occurs prior to NMJ maturation, and in the second case because presynaptic maturation is severly impaired in the mutant mice.

The knockout of several *erbB* subunit genes in mice has not provided substantial evidence in favor of a role of neuregulin receptors in activating synaptic transcription because of a probable redundancy among erbB subunits and a decrease in Schwann cell survival, which leads secondary to early nerve withdrawal. The *erbB2* and *4* genes have been inactivated by homologous recombination, but mutant animals die at embryonic day 10.5 of development because of a lack of cardiac trabeculae. The *erbB2* knockout was next genetically rescued in the heart, thus allowing the embryos to develop up to birth. In these mice, as well as in *erbB3* knockout mice, neuromuscular junctions did not show striking difference in function, ultrastructure, or composition. However, ectopic AChR clusters and reduced synaptic compartmentalization of *AChR* genes expression could be observed in the mutant animals. But whether this effect can be directly attributed to the absence of the *erbB2* or *3* gene products or to the absence of terminal Schwann cells and early nerve withdrawal is not clear (Morris et al., 1999; Woldeyesus et al., 1999). NMJ were nevertheless recently examined before retraction of the motor axon terminal in erbB2-deficient embryos, and it was found that the formation of junctional folds was significantly impaired (Lin et al., 2000). This suggests a role of erbB2 in postsynaptic development independently of its presynaptic function.

Thus, although the question remains open, neuregulins still appear as likely candidate trophic factor for the activation of synaptic genes.

A Role for Agrin and MuSK in the Control of Synaptic Gene Expression

Neuregulins are produced both by the nerve and the muscle, but have been proposed to activate transcription exclusively in subsynaptic nuclei. Consistent with this, neuregulins are only detectable in the basal lamina at the endplate, most probably because of the localization of agrin and MuSK clustering activity. There is a growing body of evidence suggesting that neuregulins are in fact acting as secondary signals, downstream of neural agrin and MuSK, which may be the primary signals in the process of activating subsynaptic transcription.

Agrin is a 210 kDa heparan sulfate glycoprotein, which was initially identified as a component of the Torpedo electric organ extracellular matrix that stimulates AChR clustering via the 43K/rapsyn protein, in muscle cell cultures (Fallon et al., 1985). Several forms of agrin are produced through alternative splicing of the *agrin* gene. The most potent isoform of agrin in AChR clustering is produced by the nerve, the muscle isoforms being less active. As shown by gene inactivation in mice and further experiments in cultured myotubes, the functional agrin receptor most probably includes the tyrosine kinase receptor MuSK (muscle specific kinase). Until recently, agrin was believed to act in a dedicated manner to promote the clustering of AChRs at the NMJ, without effects on transcriptional regulation. Yet, in knockout mice deficient for either the neural form of *agrin* or *MuSK*, subsynaptic compartmentalized expression is respectively severely affected or abolished (reviewed in Sanes and Lichtman, 1999). Normal subsynaptic transcription thus requires the presence of both neural agrin and MuSK. In addition, in vivo ectopic expression of agrin or of a constitutively active form of MuSK in muscle is sufficient to cause the formation of a functional postsynaptic apparatus, together with the upregulation of genes normally restricted to synapses (Meier et al., 1998; Jones et al., 1999).

In fact, although clearly related, agrin and MuSK seem to have distinct roles in the initiation of synapse-specific transcription. In very recent reports (Lin et al., 2001; Yang et al., 2001), early AChR distribution was investigated in mutant mice, with either noninnervated muscles or/and deficient for the *agrin*, or *MuSK*, gene. The authors made the surprising observation that AChR clustering and compartmentalized transcription takes places

at the right place in aneural muscles, thus demonstrating that the muscle is somehow prepatterned for synapse localization. In addition, MuSK, but not agrin, is required to initiate AChR clustering and local expression at embryonic day (E) 14.5 in aneural mutants. Neural agrin is nevertheless required for sustained AChR expression and clustering, since in *agrin*-deficient mice, localized AChR transcription is barely detectable at E18.5, although it was present at E14.5 (Lin et al., 2001). The most probable explanation to this phenomenon is that localized expression and transcription is initiated in muscle by MuSK activation via a still unknown mechanism. Nerve-derived agrin would then stabilize this state, protecting innervated clusters from the action of a still uncharacterized neural signal, perhaps electrical activity, promoting AChR clusters disruption (for review, see Ferns and Carbonetto, 2001).

The upregulation of synaptic gene expression in aneural muscles, or at agrin-induced ectopic synapses (Meier et al., 1998; Jones et al., 1999; Lin et al., 2001; Yang et al., 2001), shows further that neuregulins of neural origin are not required for synapse-specific transcriptional activation, since they are most probably not present in either case. Moreover, specific inactivation of the *neuregulin-1* gene in motor and sensory neurons by homologous recombination does not prevent the onset of *AChR* gene-specific synaptic expression (Yang et al., 2001). Consistently, in vitro, agrin increases the expression of the *AChR* ϵ subunit gene in cultured myotubes grown on a laminin substrate, in the absence of exogenous neuregulins (Jones et al., 1996). Two observations suggest that agrin and MuSK activate synaptic transcription via the neuregulin pathway. First, in cultured myotubes, a dominant-negative mutant of erbB2 blocks agrin elicited transcriptional activation (Meier et al., 1998). Second, in vivo, ectopic expression of agrin in muscle induces the clustering of all postsynaptic proteins including neuregulins and their receptor(s) (Meier et al., 1998; Jones et al., 1999). When considered together, the most plausible interpretation of these results is that MuSK activation induces the clustering of erbB receptors and of neuregulins, originating from either the nerve or the muscle, and that subsequent activation of downstream intracellular signaling pathways ultimately leads to transcriptional activation of synaptic genes.

Close examination of the agrin/MuSK and neuregulins/erbB receptors distribution in the postsynaptic membrane shows that agrin and MUSK are localized in the postsynaptic primary gutters together with the AChR and rapsyn, whereas neuregulins/erbB receptors are present in the depth of the secondary junctional folds. This implies that neuregulin and erbB receptor clustering induced by agrin and MuSK does not result from a direct interaction (Trinidad et al., 2000).

Signaling Pathways Involved in Synaptic Activation of Transcription

Neuregulins and their receptors are involved in the control of a wide variety of mitogenic and differentiation processes. They can therefore influence a complex network of intracellular signaling pathways, which is likely to vary with cell type and the expression of distinct erbB subunits. The signaling pathways activated in response to neuregulins have been investigated in muscle cells. Neuregulin treatment results in the phosphorylation of their erbB tyrosine kinase receptors and in the subsequent recruitment and activation of both ras and PI3 kinase. ErbB receptors also interact with another kinase, the cyclin-dependent kinase cdk5, and its activator p35. These two proteins are concentrated at the neuromuscular junction and cdk5 activity is both stimulated by, and necessary for, erbB activation by neuregulins (Fu et al., 2001). Downstream Ras activation results in the activation of the MAP kinase and JNK pathways. Blocking the MAP kinase (or only ERK2) or JNK pathways is sufficient to block neuregulin dependent activation (reviewed in Burden, 1998; Si et al., 1999). In vivo, muscle injection of dominant-negative mutants of three key components of the MAP kinase cascade—namely ras, raf, and MEK—inhibits the expression of a reporter gene driven by the *AChR* ϵ gene promoter (Si et al., 1999). The same group reported that *AChR* genes activation by neuregulins may include additional steps. In C2C12 myotubes, the stimulation of *AChR* ϵ gene expression by neuregulins is a delayed response, which requires protein synthesis and involves the expression of the immediate early genes c-*Jun* and c-*fos*. These authors also made the intriguing observation that a 60 s exposure of myotubes to neuregulins is sufficient to trigger a delayed increase in AChR ϵ mRNA level 5 hr later, and this expression reaches its maximum level 24 hr after brief stimulation. In addition, the increase of c-Jun and c-*fos* expression, as well as of ERK and JNK activities, is transient (Si et al., 1996, 1999). These observations raise the issue of the nature of the long-lasting events that are triggered by a brief application of neuregulin that manifests itself in the activation of immediate early genes, yet only 5 hr later translates into an increase in AChR subunit mRNA expression.

Although there is a general agreement about the pivotal role played by the MAP kinases, substantial discrepancies persist concerning the possible role of the PI3 kinase. In chick primary myotubes, blockade of PI3 kinase by wortmannin, or of the downstream $p70^{S6}$ kinase by rapamycin, increases the level of AChR α subunit mRNA, suggesting that the PI3 and $p70^{S6}$ kinases repress the activation by neuregulin (Altiok et al., 1997). In mouse Sol 8 cells, however, opposite results have been obtained (Tansey et al., 1996). In addition, the expression of a constitutively active form of the PI3 kinase could mimic the effect of neuregulin on the reporter gene expression driven by the AChR ϵ subunit promoter. Finally, in mouse C2C12 myotubes, the PI3 kinase pathway appears not to affect neuregulin stimulation of *AChR* gene expression (Si and Mei, 1999). These discrepancies may reflect differences in species and/or cell lines. Alternatively, they could result from differences in chromatin context or from the involvement of posttranscriptional regulation (in some cases, the endogenous mRNA levels were measured, whereas in others reporter genes were used).

Neuregulins and the N Box

Several lines of evidence argue that transcriptional activation by neuregulins is mediated by GABP and the N box. Indeed, in myotube cultures, neuregulin-mediated

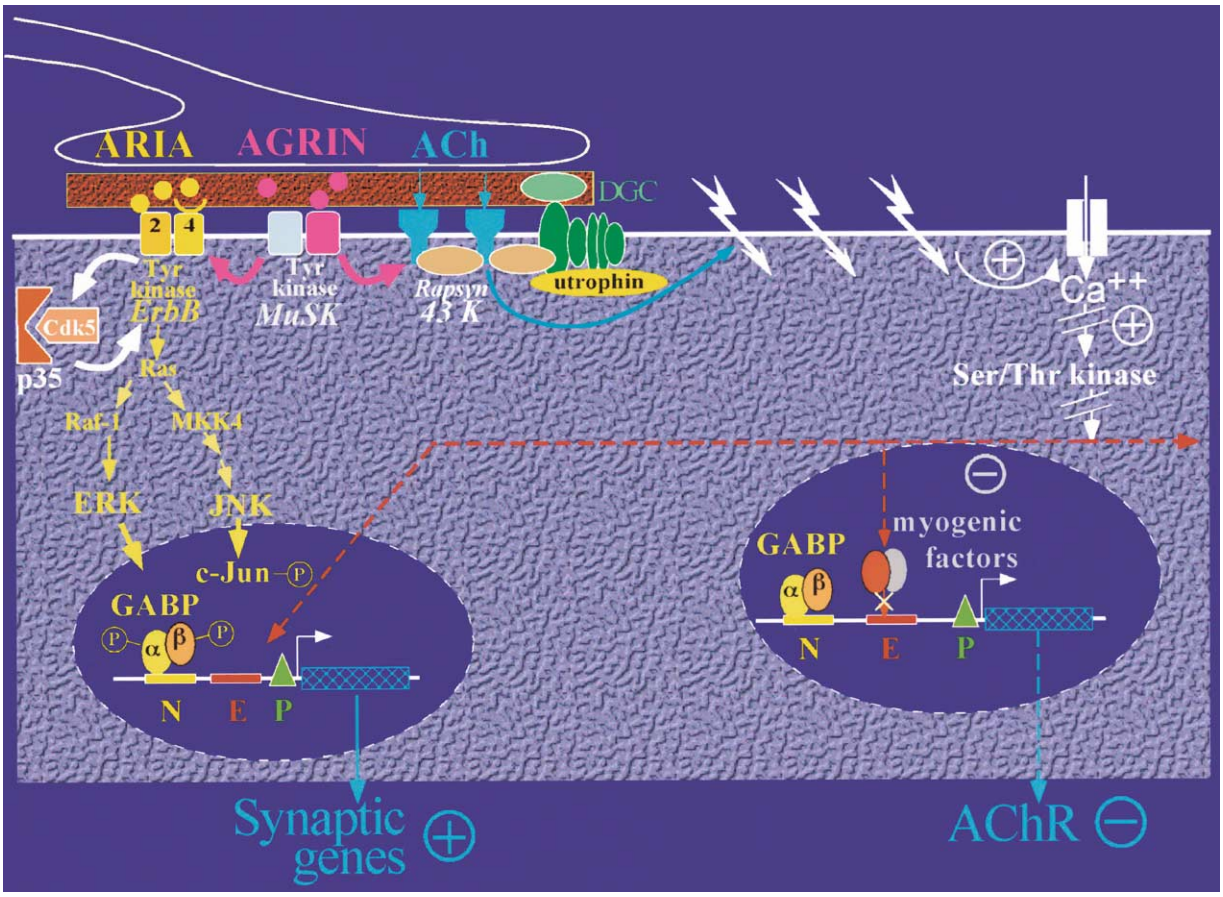

Figure 1. A Model for the Targeting of Transcription to the Neuromuscular Junction.

In subsynaptic nuclei, transcriptional activation of synaptic genes is initiated by activation of the tyrosine kinase receptor MuSK (muscle specific kinase), either as a result of autoactivation, or of the binding of the basal lamina protein agrin. Neuregulins (ARIA) and their muscle receptor, probably composed of a combination of erbB2 and erbB4, then accumulate in the basal lamina and the plasma membrane, respectively. Activation of neuregulin receptors synergizes with cdk5 and triggers both the MAP kinase and JNK pathways via Ras. This leads to the transient activation of the immediate early genes c-*Jun* and c-*fos* expression and to the phosphorylation of the Ets-related transcription factor GABP that will activate transcription of synapse specific genes via binding to the N box.

In extra synaptic nuclei, transcription of the *AChR* α, β, γ, and δ genes is severely reduced as a consequence of the AChR clustering induced by agrin, via the rapsyn/43 K protein, and of the AChR activation by acetylcholine released by the synaptic button. Indeed, nerve-evoked electrical activity in the muscle fiber triggers calcium entry into the cytoplasm, which activates serine/threonine kinases that will ultimately lead to the inhibition of the bHLH myogenic factors and thus of the *AChR* genes α, β, γ, and δ transcription (for review, see Sanes and Lichtman, 1999).

ACh, acetylcholine; ARIA, acetylcholine receptor inducing activity; GABP, GA binding protein; DGC, dystroglycan glycoprotein complex; N, N box; E, E box, binding site for the myogenic factors; P, other positive promoter elements.

activation of *utrophin*, *AChR* ϵ, or *AChR* δ promoters requires an intact N box. (Fromm and Burden, 1998; Sapru et al., 1998; Schaeffer et al.,1998; Khurana et al.,1999). Similar results have been obtained in vivo with the *utrophin* gene promoter after the stimulation of reporter gene expression by neuregulins (Gramolini et al.,1999).

The role of GABP as an intermediary in neuregulin action has been addressed using dominant-negative mutants of GABPβ and Ets-2. Such mutants, of either GABP subunits, prevents activation of the *AChR* δ and ϵ subunits promoters by neuregulin in cultured myotubes (Schaeffer et al., 1998). Overexpression of the DNA binding domain of Ets2, which blocks Ets factors binding to the N box, has a similar effect (Sapru et al., 1998). The GABP β mutant has also been expressed in vivo and prevents agrin-induced activation of the *AChR* ϵ promoter (Briguet and Ruegg, 2000). Finally, overexpression of wild-type GABP in muscle in vivo mimics the action of neuregulins on reporter gene expression through the *utrophin* gene promoter (Gramolini et al., 1999).

The finding that expression of the *utrophin* gene can be modulated by neuregulins and GABP is of potential medical interest. Utrophin is a dystrophin-related protein present only at the NMJ, where it contributes to the architecture of the postsynaptic apparatus. When expressed along *mdx* dystrophic muscle fibers, utrophin can compensate functionally for the lack of dystrophin (Tinsley et al., 1998). Therefore, the finding that neuregulin and GABP stimulate *utrophin* gene expression in vivo may have implications for the treatment of Duchenne

muscular dystrophy (DMD). These proteins may represent accessible targets for pharmacological agents designed to upregulate *utrophin* gene expression in DMD patients (Gramolini et al., 1999; Khurana et al., 1999). Studies on the molecular mechanisms that account for the targeting of gene transcription to the synapse is therefore beginning to open new avenues in the treatment and prevention of neuromuscular disorders.

If the involvement of the N box and GABP in neuregulins response is becoming clear, many of the mechanistic details of transcriptional activation by neuregulins via GABP are still obscure. Neuregulins exert several effects on GABP in cultured myotubes. They cause a 2-fold increase in GABP α levels, and they enhance the phosphorylation of both GABP subunits by MAP kinases (Schaeffer et al., 1998; Khurana et al., 1999). The neuregulin-elicited increase in GABP α level may account for the stronger expression of GABP α transcripts in synaptic nuclei (Schaeffer et al., 1998). Neuregulin-induced phosphorylation of GABP does not affect its DNA binding activity, but it may regulate the stability of the protein or its ability to activate transcription via recruitment of other transcription factors, cofactors, or by interactions with the basal transcriptional machinery.

The potential recruitment of transcription factors by GABP is particularly attractive in the light of the finding that c-Jun is required for AChR ϵ subunit stimulation by neuregulin (Si et al., 1999). Since no AP-1 site is present in the *AChR* ϵ promoters and since c-Jun recruitment by Ets transcription factors has been reported, it is possible that GABP directly recruits c-Jun to the *AChR* ϵ promoter. c-Jun protein levels have not been measured after neuregulin stimulation, but its mRNA drops to basal levels within 1 hr after stimulation. Thus, an alternative possibility is that c-Jun plays an indirect role in the stimulation of *AChR* gene expression, perhaps by controlling the induction of GABP. c-*Jun* has been inactivated by homologous recombination, but the mutant embryos die too early (at 12.5 days of development) to assess the role of c-Jun in the targeted transcription of NMJ-specific genes (Johnson et al., 1993).

Finally, a possible cooperation between transcription factors in the transduction of neuregulin signals is also supported by the finding that the promoters of the *utrophin*, as well as the δ and ϵ *AChR* genes, contain an N box in the vicinity of a CACC box and binding sites for Sp1 and bHLH factors. Indeed, the involvement of Sp-1 and CA-rich sequences in transcriptional regulation of AChRs by neuregulins has been demonstrated in P19 teratocarcinoma cells (Alroy et al., 1999).

Conclusions

The information currently available on the cellular and molecular biology of the NMJ can be expressed as a simple, albeit hypothetical, model of synapse-specific transcription in skeletal muscle (Figure 1).

The initial step in the formation of the synaptic compartment of the muscle fiber is probably MuSK activation, upon either autoactivation, activation by unknown factors, or the binding of neural agrin. These events trigger the clustering of AChRs and other molecular components of the postsynaptic apparatus involved in synaptic transmission (e.g., 43K/rapsyn, AChE, voltage-gated sodium channels). This clustering process involves the aggregation of neuregulins and their erbB receptor(s) in the synaptic basal lamina and plasma membrane, respectively.

Neuregulins stimulate cdk5 activity and the autophosphorylation of erbB receptors, which then activate Ras to trigger the two kinases Raf-1 and MEK (MAPK kinase) kinase (MEKK). This results in the rapid activation of the ERK and JNK kinases, possibly followed by activation of the immediate early gene c-*Jun* expression and the phosphorylation of both c-Jun and GABP. Phosphorylated GABP and c-Jun, either sequentially or in concert (through a still-unknown mechanism), activate transcription of the synaptic genes via GABP binding to the N box.

Agrin and neuregulins are expressed in the brain, and studies conducted on transcriptional regulation at the NMJ could be relevant for central synapses. For instance, neuregulins are involved in the induction of neuronal nicotinic acetylcholine receptors in developing chick sympathetic ganglia (Yang et al., 1998). Moreover, in cortical neuron cultures, agrin, whose neuronal receptor is still unknown, has been reported to induce immediate early gene expression, and GABA receptors clustering (Serpinskaya et al., 1999). Finally, agrin is also involved in immune synapses regulation since it is an inducer of lipid raft aggregation (Khan et al., 2001).

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