

# A novel pathway for MuSK to induce key genes in neuromuscular synapse formation

Eric Lacazette, Sophie Le Calvez, Nadesan Gajendran, and Hans Rudolf Brenner

Department of Physiology, University of Basel, CH-4056 Basel, Switzerland

At the developing neuromuscular junction the Agrin receptor MuSK is the central organizer of subsynaptic differentiation induced by Agrin from the nerve. The expression of *muskl* itself is also regulated by the nerve, but the mechanisms involved are not known. Here, we analyzed the activation of a *muskl* promoter reporter construct in muscle fibers in vivo and in cultured myotubes, using transfection of multiple combinations of expression vectors for potential signaling components. We show that neuronal Agrin by activating MuSK regulates the expression of *muskl* via two pathways: the Agrin-induced assembly of muscle-derived neuregulin (NRG)-1/ErbB, the pathway thought to

regulate acetylcholine receptor (AChR) expression at the synapse, and via a direct shunt involving Agrin-induced activation of Rac. Both pathways converge onto the same regulatory element in the *muskl* promoter that is also thought to confer synapse-specific expression to *AChR* subunit genes. In this way, a positive feedback signaling loop is established that maintains *muskl* expression at the synapse when impulse transmission becomes functional. The same pathways are used to regulate synaptic expression of *AChR $\epsilon$* . We propose that the novel pathway stabilizes the synapse early in development, whereas the NRG/ErbB pathway supports maintenance of the mature synapse.

## Introduction

The formation of a subsynaptic apparatus at the neuromuscular junction is an example of extreme subcellular differentiation induced by the motor neuron in a small segment of the muscle fiber. Four key components have been identified in this process: Agrin, a heparansulfate proteoglycan secreted from the motor nerve; MuSK, a component of the Agrin receptor complex; acetylcholine receptors (AChRs);\* and rapsyn, a peripheral membrane component required for the aggregation of the AChRs in the muscle membrane. Agrin/MuSK act upstream of AChRs and rapsyn, but deletion of any of these components prevents synapse formation (Schaeffer et al., 2001).

When a synapse forms, a group of muscle nuclei driven by signals from the nerve selectively begins to transcribe genes coding for proteins involved in synapse formation and maintenance, including *AChR* subunit genes. Two major factors expressed in motor nerves, neuregulin (NRG)-1 and Agrin,

have been invoked in this process. NRG-1, by activating ErbB receptor tyrosine kinases in the muscle membrane, is thought to activate *AChR* genes (Burden and Yarden, 1997; Fischbach and Rosen, 1997). Agrin by activating the receptor tyrosine kinase MuSK aggregates the AChRs and MuSK in the membrane. In cultured myotubes, activation of ErbB receptors by NRG-1 activates *AChR $\alpha$* ,  $\delta$ , and  $\epsilon$  subunit gene transcription via the MAPK (extracellular signal-regulated kinase [ERK]) and the phosphatidylinositol 3'-kinase pathways (Tansey et al., 1996; Altiok et al., 1997). ERK in turn activates the Ets transcription factor GABP to bind to a regulatory element in the *AChR $\epsilon$*  and  $\delta$  promoters, termed N-box, thus stimulating the transcription of the respective genes. The same regulatory element also mediates the localization of *AChR $\delta$*  and  $\epsilon$  subunit expression to the synapse (Koike et al., 1995; Duclert et al., 1996; Fromm and Burden, 1998; Schaeffer et al., 1998; Sapru, 2001). Together these data suggest that the nerve regulates synaptic *AChR* expression via NRG-1. However, NRG-1 from neurones is not required to induce *AChR* genes in muscle fibers. Specifically, activation of MuSK by Agrin induces the formation of a postsynaptic membrane including *AChR* expression in vivo in the absence of a nerve terminal and thus of neural NRG-1 (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997).

The mRNA for MuSK also accumulates at the synapse, suggesting synapse-specific activation of its gene by the nerve (Valenzuela et al., 1995), but the mechanisms involved are

Address correspondence to Hans Rudolf Brenner, Dept. of Physiology Biozentrum/Pharmazentrum, University of Basel, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland. Tel.: 41-61-267-16-38. Fax: 41-61-267-16-28. E-mail: Hans-Rudolf.Brenner@unibas.ch

\*Abbreviations used in this paper: AChE, acetylcholinesterase; AChR, acetylcholine receptor;  $\beta$ -Gal,  $\beta$ -galactosidase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; NRG, neuregulin.

N. Gajendran's present address is Max-Planck-Institut für Infektionsbiologie, Abt. Immunologie, Schumannstrasse 21/22, D-10117 Berlin, Germany.

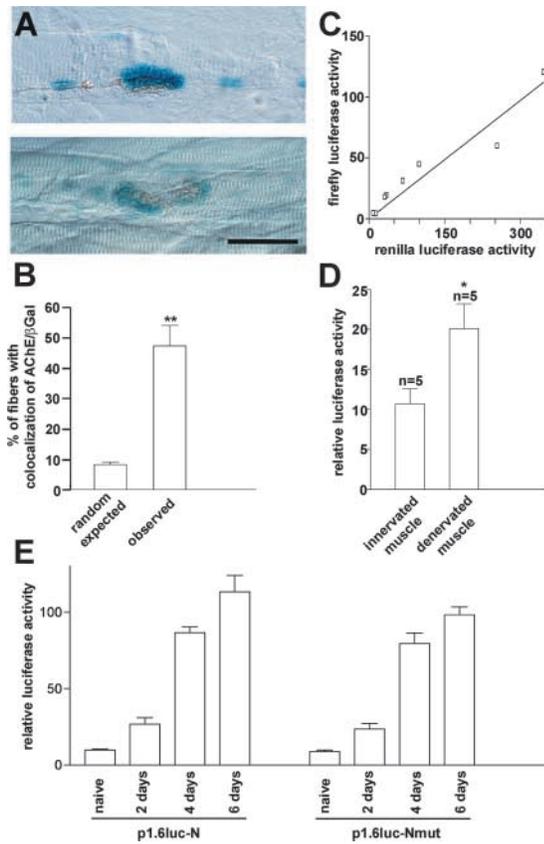
Key words: Agrin; MuSK; synapse formation; skeletal muscle; neuromuscular junction



To test for synapse-specific activation, the proximal 1.6-kb upstream region was linked to *nlslacZ* as a reporter, followed by 0.5 kb of intronic sequence containing the N-box (Fig. 1 C, p1.6nlslacZ-N). Innervated, i.e., electrically active rat soleus muscles were then transfected in vivo by electroporation with p1.6nlslacZ-N, and synapse-specific expression of *nlslacZ* was tested 10–14 d later in the microscope by examining the location of muscle nuclei expressing  $\beta$ -galactosidase ( $\beta$ -Gal)-positive nuclei with respect to the location of synapses. The latter were marked by histochemical stain-

ing for acetylcholinesterase (AChE). Indeed, in some fibers  $\beta$ -Gal-positive nuclei were tightly colocalized with synapses (Fig. 2 A); in other fibers they were also seen in nonsynaptic regions. To see whether the AChE/ $\beta$ -Gal colocalization reflected preferential activation of the *nsk2/musk* promoter fragment at the synapse, we compared the number of  $\beta$ -Gal/AChE colocalizations expected for a random process with the number of colocalizations actually observed. For this purpose, fiber bundles were dissected, and the lengths of fiber segments occupied by  $\beta$ -Gal-positive nuclei relative to the length of the dissected fibers were determined. This ratio multiplied by the number of  $\beta$ -Gal-positive fibers examined gives the number of  $\beta$ -Gal-positive synapses expected for random colocalization. Comparison with the number of  $\beta$ -Gal/AChE colocalizations actually observed showed that  $\beta$ -Gal-positive synapses occurred five to six times more frequently than expected by chance (Fig. 2 B).

To test the *nsk2/musk* promoter fragment for its activation by muscle denervation, *nlslacZ* in p1.6nlslacZ-N was replaced by the cDNA encoding firefly luciferase (*luc*) as a reporter (Fig. 1 C, p1.6luc-N). Innervated, i.e., electrically active rat soleus muscles were then electroporated in vivo with a mixture of p1.6luc-N and an expression vector for Renilla luciferase, pRL-TK, as a reference. 10–13 d later, the soleus nerve was cut. After another 4 d, luciferase activities normalized to RL-TK activity in muscle extracts were compared with those in transfected control fibers that had been left innervated. The comparison revealed a significant twofold increase in normalized luciferase activities upon denervation (Fig. 2 D). A similar increase was seen when the N-box was mutated as described in the next paragraph, indicating that up-regulation by denervation was not dependent on the N-box. Finally, the expression profile of p1.6luc-N during in vitro differentiation of C2C12 muscle cells was similar to that of endogenous *musk* mRNA in these cells, i.e., a low level in myoblasts followed by a strong increase in differentiating myotubes (Valenzuela et al., 1995), and it was not affected by mutations in the N-box (Fig. 2, E and F). Thus, the isolated 2.1-kb promoter fragment of *nsk2/musk* confers expression patterns to the reporters as they are observed for endogenous *musk*.



**Figure 2. A 2.1-kb *nsk2/musk* promoter fragment confers spatio-temporal expression patterns to reporters comparable to those of endogenous *musk*.** (A) Examples of endplates marked by histochemical staining for AChE and expressing colocalized  $\beta$ -Gal-positive nuclei upon transfection with p1.6nlslacZ-N. Nuclei are in blue and AChE is in brown. Bar, 40  $\mu$ m. (B) The percentage of  $\beta$ -Gal-positive fibers with colocalizations of  $\beta$ -Gal-positive nuclei and synaptic AChE observed (as illustrated in A) is significantly higher than expected for a random process. Means  $\pm$  SE are given ( $n = 4$  muscles, 17–56  $\beta$ -Gal-positive fibers [170 fibers total] examined per muscle). (C) The level of firefly luciferase (*luc*) activity expressed upon transfection with p1.6luc-N increases linearly with the activity of a cotransfected standard, p(RL-TK), of Renilla luciferase, indicating that normalization to RL-TK expression to account for differences in transfection efficiency between muscles as performed in D is appropriate. (D) The 2.1-kb *nsk2/musk* promoter fragment responds to muscle denervation (4 d) by increasing expression of luciferase activity (means  $\pm$  SE,  $n = 5$ ). In the same experiments, the levels of RL-TK were not significantly different in innervated control and in denervated fibers (data not depicted). (E) Activation of p1.6luc-N and p1.6luc-Nmut in C2C12 myoblasts and in differentiating myotubes ( $n = 3$  parallel cultures for each transfected plasmid). \* $P < 0.05$  and \*\* $P < 0.01$  in two-tailed *t* test. Bars  $\pm$  SEM.

### Agrin activates the 2.1-kb *nsk2/musk* fragment conferring synapse-specific expression in vivo via an N-box

Expression of endogenous *musk* can be induced by Agrin/MuSK (Moore et al., 2001). Therefore, we next tested whether the 2.1-kb *nsk2/musk* promoter fragment conferring synaptic expression could be activated specifically by neuronal Agrin. For this purpose, we injected expression plasmids coding for full-length chicken neuronal Agrin, pcAgrin748, p1.6luc-N, and pRL-TK as a reference along with pnlsGFP-S6 intracellularly into extrasynaptic regions of innervated soleus muscles. As a control, pcAgrin748 was replaced with an expression vector for the nonneuronal isoform of Agrin, pcAgrin700 (Ruegg et al., 1992). 2 wk later, in fiber regions containing GFP-positive nuclei, luciferase activity measured in fibers expressing neuronal Agrin was  $\sim 3.5$  times higher than in fibers expressing muscle Agrin (Fig. 3 A). On a “per nucleus” basis, this is a lower estimate because the fiber segments dissected (2–3 mm in length)

contained many more GFP-positive nuclei outside the Agrin-induced ectopic, postsynaptic membranes (and thus not activated by Agrin) than nuclei colocalized with such sites. Thus, neuronal Agrin but not muscle Agrin activates the same 2.1-kb *nsk2/musk* promoter fragment, (1.6 N) as does the nerve.

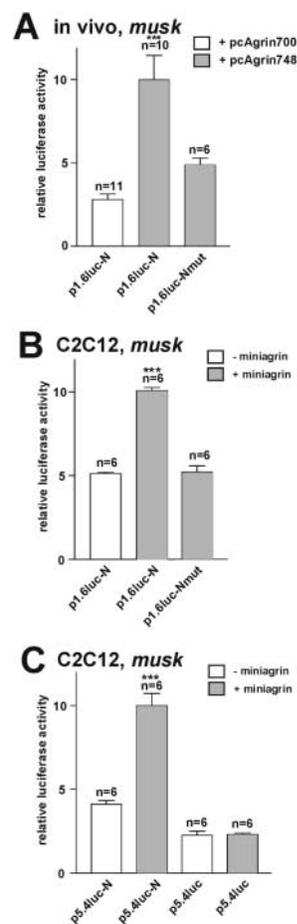
Unlike during myotube differentiation and upon denervation, the N-box of *musk* was essential for the activation of the *musk* promoter by Agrin. When two nucleotides in the N-box were mutated (CCGGAA → TTGGAA) to produce p1.6luc-N<sub>mut</sub>, agrin-induced luciferase activity was significantly reduced (Fig. 3 A).

### Neuronal Agrin triggers N-box–dependent *musk* expression in C2C12 myotubes

For further characterization of the *nsk2/musk* promoter, the responsiveness to Agrin of different promoter fragments of various lengths was examined by transactivation assays in C2C12 myotubes. In these experiments, a minimal variant of recombinant neuronal Agrin, cAgrinNtAc21B8 (Meier et al., 1998a), was presented to the myotubes in a form attached to the laminin culture substrate. Indeed, p1.6luc-N showing physiological activation in vivo also responded to neuronal mini-Agrin in myotubes but only when the N-box was intact (Fig. 3 B). Similar results were obtained when, immediately upstream from the transcription start site, the promoter was reduced to 0.2 kb (p0.2luc-N; unpublished data) or when it was extended to contain 5.4 kb of upstream sequence (p5.4luc-N; Fig. 1 C), except that basal luciferase activities and the response to Agrin increased somewhat as the upstream fragment length was increased (unpublished data). However, in all cases constructs required the presence of the intronic N-box to respond to Agrin (Fig. 3 C).

### Binding of inactive GABP to the N-box blocks activation of *nsk2/musk* by Agrin

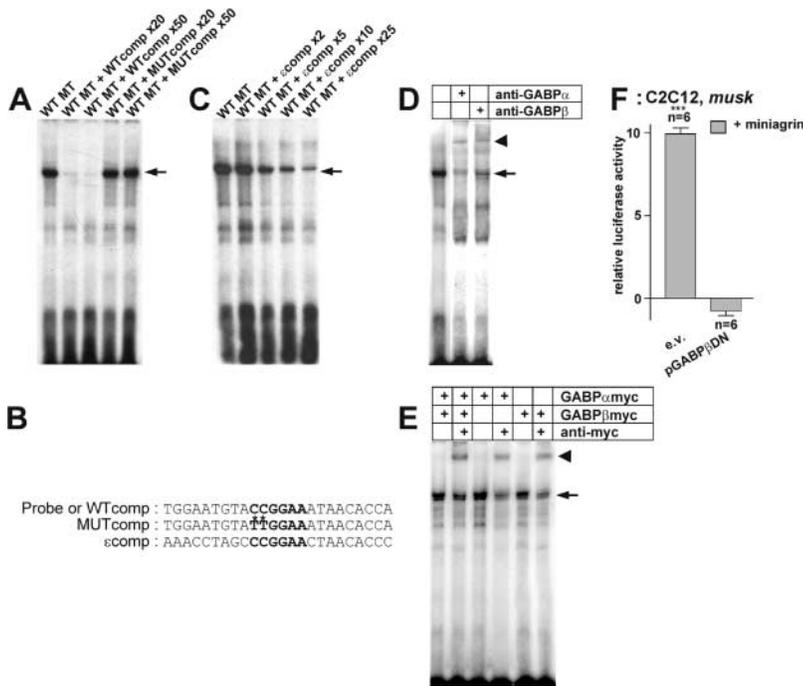
In cultured myotubes, *musk*, like *AChR*, is also activated by NRG-1 (Ip et al., 2000). The NRG-dependent activation of *AChR* genes depends on the binding of active transcription factor GABP to the N-box (Koike et al., 1995; Duclert et al., 1996; Fromm and Burden, 1998; Schaeffer et al., 1998). Therefore, we next tested in mobility shift assays whether the transcription factor complex GABP, composed of GABP $\alpha$ - and  $\beta$  subunits (Batchelor et al., 1998), binds to the N-box of the *nsk2/musk* promoter fragment, using a radiolabeled 24-base fragment of the first intron containing the N-box motif as a probe. Exposure of the probe to nuclear extracts from C2C12 myotubes revealed binding activity in the extract (Fig. 4 A). Adding nonlabeled probe eliminated the detectable shifted complex, indicating specificity of binding. Integrity of the N-box was necessary, since the nonradiolabeled probe with the N-box mutated was ineffective in competing for binding of the nuclear extract (Fig. 4, A and C). We next examined whether addition of a 24-nucleotide fragment of the rat AChR $\epsilon$  subunit promoter, which also contains an N-box,  $\epsilon$ -comp (Fig. 4 B), could compete for binding activity from C2C12 nuclear extracts. Indeed, a 25-fold molar excess of  $\epsilon$ -comp prevented the retardation of the labeled N-box probe from *nsk2/musk* that



**Figure 3. Neuronal but not muscle agrin activates *nsk2/musk* promoter fragments, depending on an N-box in the first intron.** (A) Expression of luciferase activity under the control of the (1.6-N) promoter fragment in adult soleus muscle fibers in vivo is promoted by neuronal but not my muscle Agrin and is blocked upon mutation of the N-box. Muscle fibers were injected intracellularly with the expression plasmids indicated and with pRL-TK for normalization. (B) Agrin-induced expression of luciferase under the control of various *nsk2/musk* promoter fragments depends on the presence of the N-box in C2C12 myotubes. C2C12 myoblasts were transfected with pRL-TK and the plasmids indicated and grown and differentiated on a laminin substrate with or without mini-Agrin N25;c21B8 attached. (C) Agrin-induced activation of a 5.4-kb *nsk2/musk* promoter fragment in C2C12 myotubes is blocked by removal of the intronic sequence containing the N-box. \*\*\* $P < 0.001$  in two-tailed  $t$  test. Bars  $\pm$  SEM.

had been produced by the addition of C2C12 nuclear extract (Fig. 4 C). Furthermore, a supershift was produced by binding of anti-GABP $\alpha$  or anti-GABP $\beta$  antibodies (Fig. 4 D). Likewise, when the labeled probe was exposed to an extract from HEK293 cells transiently expressing myc-tagged GABP $\alpha$  and GABP $\beta$ , respectively, supershifts were observed in the presence of anti-myc antibody (Fig. 4 E). These data combined indicate that the N-box motif of the MuSK promoter specifically binds GABP.

Next, we tested whether Agrin-induced *musk* activation was abolished by binding of an inactive mutant of GABP. For this purpose, we compared in C2C12 myotubes the response of p1.6luc-N to Agrin in the presence and in the absence of pGABP $\beta$ DN, an inactive mutant of GABP $\beta$ ,



**Figure 4. GABP binds to the N-box of a *nsk2/musk* promoter fragment and activates it.** (A) Binding activity of wild-type nuclear extracts to the N-box present in the probe. The specificity of the signal is eliminated by competition with an unlabeled wild-type probe (WTcomp), whereas an unlabeled mutant competitor (MUTcomp) leaves the signal unaffected. (B) Sequences of the oligonucleotides used as probes or competitors. (C) Unlabeled oligonucleotide containing the N-box motif of the *AChR $\epsilon$*  subunit promoter ( $\epsilon$ -comp) competes with N-box of *muskl* for binding of nuclear extract. (D) The N-box binding activity produced by nuclear extracts from C2C12 cells (lane 1) is supershifted (bold arrow) by antibodies directed against GABP $\alpha$  (lane 2) and GABP $\beta$  (lane 3). (E) Nuclear extracts of transiently transfected HEK293 cells expressing GABP $\alpha$ -myc and GABP $\beta$ -myc (lanes 1 and 2) or only GABP $\alpha$ -myc (lanes 3 and 4) or GABP $\beta$ -myc (lanes 5 and 6) possess an N-box binding activity (arrow), which is supershifted (bold arrow) in the presence of an anti-myc antibody (Ab, lanes 2, 4, and 6). The second band seen in the shift may originate from binding activity specific for HEK293 cells. (F) Agrin-induced activation of the 2.1-kb *nsk2/musk* promoter fragment in C2C12 myotubes is blocked by overexpression of GABP $\beta$ DN. \*\*\*P < 0.001 in two-tailed t test. Bars  $\pm$  SEM. Note that for better resolution of the agrin-specific induction, luciferase activities in parallel control cultures (not depicted) are subtracted.

which blocks activation of *AChR* genes in cultured myotubes (Schaeffer et al., 1998) and Agrin-induced postsynaptic differentiation in vivo (Briguet and Rüegg, 2000). Indeed, overexpression of GABP $\beta$ DN abolished the induction of luciferase activity under the 1.6-N promoter fragment by mini-Agrin (Fig. 4 F). In fact, luciferase activity in Agrin-exposed, GABP $\beta$ DN-overexpressing cultures were below those in Agrin-free control cultures, suggesting some constitutive activity of GABP or a similar factor in the absence of Agrin. Thus, induction of *muskl* expression by neuronal Agrin involves binding of GABP or a related factor to the intronic N-box.

### Agrin can activate the *nsk2/musk* promoter via two pathways converging on the N-box

In cultured myotubes, NRG-1/ErbB-induced activation of *AChR $\delta$*  and  $\epsilon$  is mediated by ERK and JNK MAPKs (Tansy et al., 1996; Altioek et al., 1997; Si et al., 1999). Therefore, we examined whether *nsk2/musk* was induced indirectly by Agrin, i.e., via the organization of an NRG-1/ErbB pathway with NRG derived from the myotubes, as we had observed previously for the induction of *AChR $\epsilon$*  by Agrin (Meier et al., 1998b). As predicted for such a mechanism, activation of p1.6luc-N by mini-Agrin in C2C12 myotubes was abolished by overexpression of pHER2KM or pHER4KM, dominant-negative mutants of ErbB2 and ErbB4 (Fig. 5 A). Activation of *nsk2/musk* by Agrin was also blocked by overexpression of a dominant-negative mutant of JNK, pJNK-APF (Fig. 5 B). Conversely, a constitutively active mutant of MKK7, pMKK7D, which activates JNK, induced *nsk2/musk* in the absence of Agrin. A promoter-reporter construct of *AChR $\epsilon$*  (Jones et al., 1996) was regu-

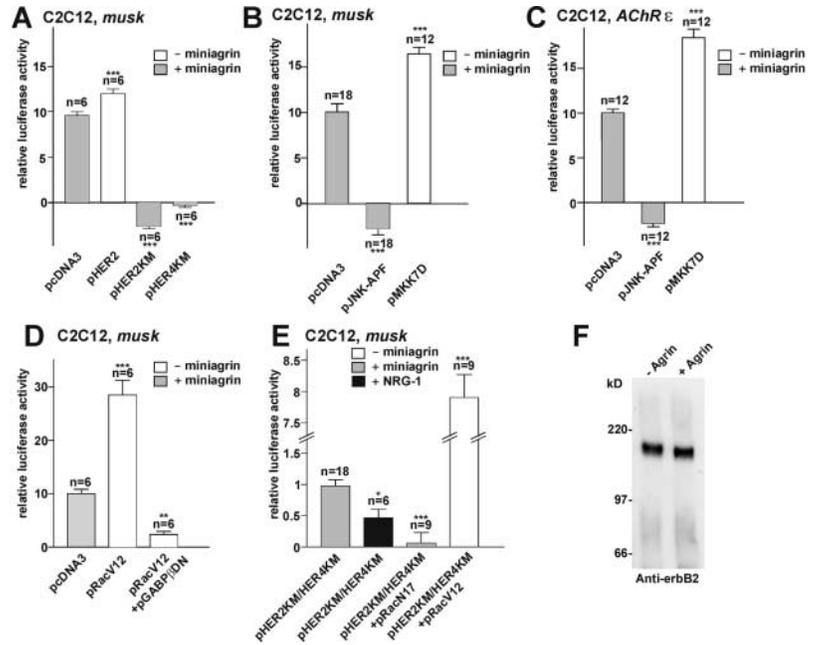
lated similarly (Fig. 5 C). These data suggest that Agrin organizes a secondary NRG-1/ErbB pathway, which in turn activates *muskl* and *AChR $\epsilon$*  via ERK and JNK.

Next, we asked whether Agrin could also activate *muskl* via a direct shunt from MuSK. For example, an alternative way for Agrin to activate JNK in C2C12 cells is via the Rho family GTPases Rac and Cdc42 (Weston et al., 2000; Luo et al., 2002). Consistent with such a mechanism, constitutively active Rac, RacV12, strongly activated p(1.6luc-N) in the absence of Agrin. This effect was abolished by GABP $\beta$ DN (Fig. 5 D), suggesting that Rac activates *nsk2/musk* via the N-box only. If Agrin-induced activation of *muskl* via Rac occurs independently of NRG/ErbB, Agrin will induce p1.6luc-N more strongly than saturating concentrations of NRG-1, and induction will be blocked by a dominant-negative mutant of Rac, RacN17. In normal C2C12 myotubes, no difference in the activation of p1.6luc-N by Agrin and by NRG-1 was resolved (unpublished data). However, when ErbB signaling was blocked by overexpression of inactive ErbB mutants, Agrin activated p(1.6luc-N) more strongly than saturating concentrations of NRG-1 (2 nM), which was not due to increased levels of ErbB2 (Fig. 5 F). Furthermore, activation was completely blocked by RacN17 (Fig. 5 E). Together, these data suggest that Agrin/MuSK can activate *nsk2/musk* via two pathways (Fig. 7): (a) via a direct shunt activated by MuSK involving Rac and (b) via the organization of a secondary NRG-1/ErbB pathway. Both pathways converge onto GABP. In C2C12 myotubes, the secondary NRG-1/ErbB pathway occludes the MuSK-induced shunt.

Next, we examined whether these pathways could be resolved in muscle fibers in vivo. Unlike in C2C12 cells, activation of *nsk2/musk* by Agrin was not blocked in adult fibers

**Figure 5. Agrin uses two pathways for the activation of the 2.1-kb *nsk2/musk* promoter fragment in cultured myotubes.**

(A) In C2C12 myotubes, activation of p1.6luc-N by Agrin is fully blocked by HER2KM and HER4KM, dominant-negative mutants of human ErbB2 and ErbB4, respectively, whereas wild-type HER2 mimics the induction by Agrin. (B) Same as in A but blockade by dominant-negative JNK, JNK-APF; conversely, overexpression of MKK7D, a constitutively active kinase activating JNK, activates the fragment in the absence of Agrin. (C) Same as in B but with an *AChR $\epsilon$*  promoter fragment (pLCF216 $\epsilon$ ). Note similar activation patterns of *nsk2/musk* and *AChR $\epsilon$*  promoter fragments. (D) Constitutively active Rac, RacV12, activates p1.6luc-N; induction by Rac is completely blocked by GABP $\beta$ DN. (E) With ErbB signaling blocked, p1.6luc-N is activated more strongly by agrin than by saturating (2 nM) NRG-1, and responses are fully blocked by dominant-negative Rac, RacN17. Constitutively active Rac, RacV12, strongly activates the promoter in spite of blockade of ErbB signaling. All experiments were performed on C2C12 cells transfected with p1.6luc-N. (F) Agrin does not discernibly increase ErbB2 levels in C2C12 myotubes. Experiments performed as in Fig. 3 with plasmids transfected as indicated. Responses are normalized to control values in the left-hand columns, and nonspecific background luciferase activities as observed in myotubes on agrin-free substrate are subtracted (C). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , respectively, in two-tailed  $t$  test. Bars  $\pm$  SEM.



by overexpression of the inactive ErbB mutants. Specifically, upon injection with pHER2KM and pHER4KM into muscle fibers in vivo, activation of p1.6luc-N by neuronal Agrin was not significantly reduced compared with fibers injected with wild-type pHER2 and pHER4 (Fig. 6 A). Expression of pHERKM and accumulation of HER2KM at ectopic sites was ascertained by immunocytochemistry (Fig. 6 B). Similar results were obtained with the *AChR $\epsilon$*  promoter-reporter construct (unpublished data).

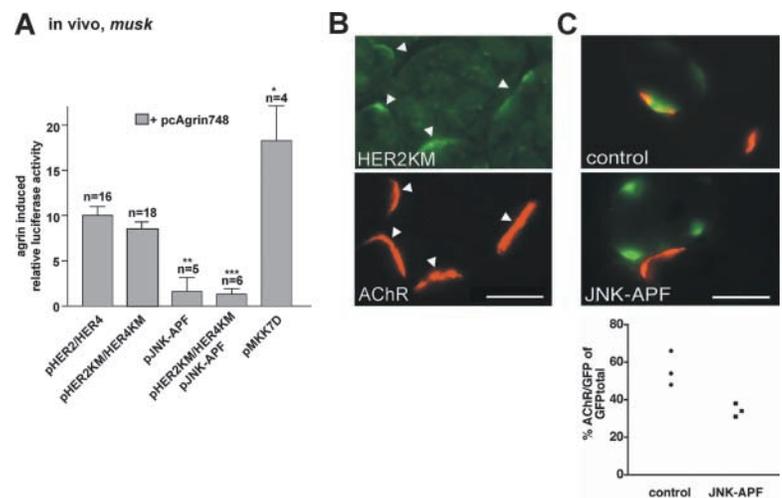
In contrast, overexpression of the inactive JNK mutant, JNK-APF, did block *musk* activation by Agrin (Fig. 6 A).

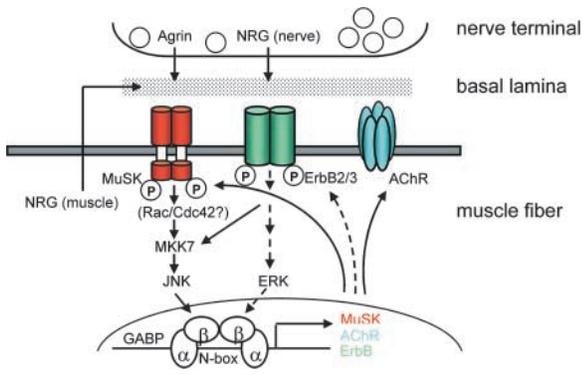
Furthermore, with JNK-APF overexpressed the presence of HER2KM and HER4KM did not inhibit the promoter activation any further. Conversely, coinjection of pMKK7D significantly increased *musk* activation by Agrin in muscle fibers. Therefore, in adult fibers Agrin/MuSK induces *nsk2/musk* primarily via a direct shunt involving Rac and JNK activation but not NRG/ErbB signaling.

If active JNK activation promotes gene expression at the synapse, then inactive JNK-APF is predicted to inhibit Agrin-induced expression of endogenous synaptic genes, such as *musk* and *AChR $\epsilon$* . We tested this prediction in vivo

**Figure 6. Agrin activates *nsk2/musk* via an ErbB-independent pathway in muscle fibers in vivo.**

(A) Activation of the *nsk2/musk* promoter fragment by Agrin in vivo is marginally affected by HER2KM and HER4KM but is fully blocked by JNK-APF. MKK7D activates the *nsk2/musk* fragment in the absence of Agrin. Nonspecific background luciferase activities as observed in muscle fibers injected with expression vector for muscle agrin (pcAgrin700; see Fig. 3 A) are subtracted. (B) Injection of pHERKM induces expression of HERKM in muscle fibers in vivo as myc immunoreactivity was observed at agrin-induced ectopic postsynaptic membranes upon injecting pmc\_HER2KM, a fusion of HER2KM and myc. Bar, 25  $\mu$ m. (C) JNK-APF inhibits ectopic subsynaptic differentiation by Agrin. Muscle fibers were injected with pcAgrin748, pnsGFP, and pJNK-APF or equivalent amounts of empty vector (control); the formation of ectopic AChR clusters in GFP-positive fibers was then examined in cross sections, and injected fibers were identified by their expression of GFP. (Top) Control fiber (note the formation of AChR cluster [red] in GFP-positive control fiber [green nuclei]). (Middle) Agrin secreted induces AChR cluster on adjacent, GFP-negative (JNK-APF-negative) but not on GFP- (JNK-APF) expressing fiber. Bar, 25  $\mu$ m. (Bottom) The percentage of GFP-positive fibers forming AChR clusters in response to Agrin is depressed in JNK-APF compared with control fibers. Each data point represents one muscle. 83–513 fiber profiles were examined per muscle.





**Figure 7. Model for stabilization of synaptic gene expression through stabilization of *musk* expression by agrin from motor nerve terminal.** Agrin secreted from nerve terminal activates preexisting MuSK to induce expression of *musk* via its N-box (i) by organizing an NRG/ErbB pathway, involving MuSK-induced recruitment of ErbB receptors and of muscle-derived NRG and (ii) by MuSK-induced activation of JNK (via Rac/Cdc42). With *musk* expression stabilized, the same pathways are used for AChR and *erbB* expression. Expression may be strengthened by NRG-1 secreted from nerve terminal. Complete inhibition of Agrin-induced *musk* transcription in C2C12 cells by overexpression of inactive ErbBs (HER2KM and HER4KM) and by dominant-negative JNK suggests that the two pathways are connected. The model is based on present data (solid arrows) and references cited (broken arrows).

by analyzing whether JNK-APF blocked Agrin-induced ectopic subsynaptic differentiation. Indeed, injection of pJNK-APF significantly reduced the formation of ectopic AChR clusters in electrically active fibers (Fig. 6 C). Since overexpression of inactive JNK does not affect AChR clustering itself (Luo et al., 2002), the inhibition is due to down-regulation of expression of genes important in synapse formation, such as *musk* and *AChR*.

## Discussion

To understand how the motor neuron regulates the formation of a subsynaptic muscle membrane, it is important to know how it regulates expression of MuSK, the central organizer of subsynaptic differentiation. Endogenous *musk* is stimulated in vivo by activated MuSK (Moore et al., 2001), but the pathways involved are not known. Here, we have used various *nsk2/musk* promoter-reporter constructs comprising up to 5.4 kb upstream and 0.5-kb sequence from the first intron to analyze the signaling pathways activated by the nerve and by Agrin in muscle fibers in vivo and in cultured myotubes.

Using these constructs, we show the following: (a) the nerve can induce activation of *nsk2/musk* promoter fragments via pathways converging on a regulatory sequence termed N-box in the first intron of *musk*; (b) Agrin activates the *nsk2/musk* promoter fragments solely via the binding of an Ets transcription factor to this N-box; (c) activation is mediated by two converging pathways, the organization of an NRG-1/ErbB pathway and a novel NRG-1/ErbB-independent pathway via MuSK/Rac; (d) the NRG-1/ErbB-mediated pathway predominates in cultured myotubes, the other in muscle fibers in vivo; and (e) these pathways are also used for the regulation of an *AChRE* promoter fragment.

Recently, it has been proposed that NRG may regulate *AChRE* and *AChR $\delta$*  subunit genes via Sp1 binding to a neuroregulin response element (CCACCCCC) different from the N-box in their promoters (Alroy et al., 1999). The p5.4luc-N construct contains at approximately  $-3.4$  kb a consensus sequence of this type. However, neither this putative neuroregulin response element nor an Sp1-binding consensus sequence at position  $-112$  in the *musk* promoter rescued the construct's responsiveness to Agrin when it was lacking the N-box, indicating that these sequences are not involved in the regulation of *musk* by Agrin.

An important aspect of the physiological relevance of the exogenous promoter fragment is whether it reflects the expression pattern of endogenous *musk* in vivo. The 2.1-kb *nsk2/musk* fragment used for most experiments here satisfies this criterion, except that the specificity of the reporter expression at the synapse and its relative increase upon denervation were less pronounced. This was likely due to nonspecific activation of the transfected promoter fragment in extrasynaptic fiber segments, perhaps caused by a high number of plasmid molecules introduced into the fibers or by the absence of regulatory elements that are located beyond this region.

In cultured myotubes, the organization of a secondary NRG-1/ErbB pathway by Agrin is suggested by the blockade of Agrin-induced *nsk2/musk* promoter activation upon inhibition of ErbB-mediated signaling as found previously for *AChRE* (Meier et al., 1998b). Induction of *AChRE* via NRG is mediated by ERK (Tansey et al., 1996; Altioek et al., 1997) and involves activation of JNK MAPKs (Si et al., 1999). Specifically, although inactive JNK blocks NRG-induced *AChRE* activation, overexpression of wild-type JNK has no effect, suggesting that JNK is necessary but not sufficient to induce *AChRE*, but requires ERK (Si et al., 1999). However, the present data suggest that JNK can activate *musk* independently of NRG/ErbB. (a) In spite of ErbB signaling blocked, *musk* was activated by constitutively active RacV12 (Fig. 5 E). Interestingly, JNK is activated by Agrin/MuSK in C2C12 myotubes via Rac (Weston et al., 2000) and via Dishevelled (Luo et al., 2002). (b) With ErbB signaling blocked, *musk* activation by Agrin was stronger than by saturating concentrations of NRG, and activation was completely blocked by dominant-negative RacN17. Therefore, these findings suggest that in addition to the NRG/ErbB pathway Agrin activates an ErbB-independent shunt via Rac/JNK to induce *musk*. Both converge on GABP because GABP binds to the N-box, and its inactive mutant GABP $\beta$ DN completely blocks ErbB- and Rac-dependent activation of *musk*. One explanation for the prevalence of the ErbB-dependent pathway in C2C12 cells may be the high levels of constitutively expressed NRG-1 and ErbBs in these cells (Meier et al., 1998b) which could favor the organization of the NRG/ErbB pathway.

In contrast, in muscle fibers in vivo, *musk* induction by Agrin is only marginally mediated by NRG/ErbB because it is little affected by HER2KM and HER4KM. Thus, Agrin appears to activate *musk* primarily via the Rac/JNK-dependent shunt pathway: (a) only interfering with JNK but not abolishing NRG/ErbB signaling abolished Agrin-induced *musk* expression, and (b) forced overexpression of MKK7, which activates JNK but not ERK and p38 MAPKs (Davis,

2000), induced *musk*, again suggesting convergence of the NRG/ErbB and the shunt pathways.

JNK is a plausible candidate mediating Agrin's induction of *musk*. It can phosphorylate GABP in vitro (Fromm and Burden, 2001) and in T cells where it induces interleukin-2 (Hoffmeyer et al., 1998). Thus, Agrin-induced *musk* activation mediated by JNK will depend on the presence of the N-box in the *musk* promoter as observed here. Consistent with a role in Agrin-dependent gene transcription, JNK-APF inhibited ectopic subsynaptic differentiation as assessed by the inhibition of ectopic AChR cluster formation induced by Agrin. A similar blockade has been found by dominant-negative GABP $\beta$ DN with the same type of assay (Briguet and Rüegg, 2000).

Our conclusion of an ErbB-independent, MuSK-induced shunt prevailing in vivo depends on the functional expression of injected pHERKM in the muscle fibers. Evidence for this is (a) the immunohistochemical demonstration of their expression; (b) that pHER2KM and pHER4KM have been functionally tested in HEK293 cells (Weiss et al., 1997); (c) that transfection of pHER2/4KM into C2C12 cells blocks *musk* and *AChR $\epsilon$*  activation by Agrin, and thus, the constructs are functional in muscle cells; (d) that injection of pHER4KM into muscle fibers in vivo partially inhibits the induction of ectopic synaptic membranes by Agrin; inhibition was similar to that caused by ablation of the *erbB2* gene (Moore et al., 2001). The inhibitions by *erbB2* ablation and by pHER4KM injection were incomplete but similar in magnitude, and they did not add. Thus, injection of pHER4KM alone could fully block NRG/ErbB signaling in vivo. As predicted for an NRG/ErbB-independent pathway, the blocking effect by ErbB ablation and HER4KM overexpression was reduced as the level of Agrin was increased.

A direct shunt from MuSK to the nuclei is consistent with the only marginal reduction in AChR density that we observed recently at mature endplates of *erbB2*<sup>-/-</sup> muscle fibers (Leu et al., 2003). Finally, it can explain the observation that in neonatal *rapsyn*<sup>-/-</sup> mice elevated levels of *musk* and *AChR $\gamma$*  and  $\delta$  subunit genes are expressed in the synaptic muscle region (Apel et al., 1997), although MuSK but not ErbB receptors are aggregated (Moscoso et al., 1995).

Recently, de Kerchove D'Exaerde et al. (2002) reported that unlike *AChR $\epsilon$*  subunit mRNA, the level of endogenous *musk* mRNA was not reduced in transgenic mouse muscle expressing an inactive mutant of the transcription factor Ets2. They concluded that *musk* is not or only marginally regulated by a mechanism involving Ets transcription factors, in contrast to the findings described here. However, alternative explanations are also possible (see Discussion in de Kerchove D'Exaerde et al., 2002).

By stabilizing *musk* and, as a consequence, *AChR* and other genes, Agrin can "stabilize" developing neuromuscular junctions: in fetal muscle, postsynaptic-like sites begin to develop in the absence of Agrin but depend on the presence of MuSK; later in development, they become dispersed, unless they are stabilized by Agrin from motor nerve terminals (Lin et al., 2001; Yang et al., 2001). Apparently, NRG from nerve terminals is not sufficient to stabilize MuSK. At mature synapses, NRG-1 secreted from the nerve is likely to contribute more substantially to the synaptic expression of

*musk* because more NRG-1 will be available than can be recruited by Agrin from the muscle fibers alone (Loeb et al., 1999). Consistent with this, mice heterozygous for the relevant NRG-1 isoform contain fewer AChRs than wild-type mice in their endplates (Sandrock et al., 1997); similarly, in wild-type mice Agrin-induced ectopic AChR clusters have lower AChR density than endplates.

Finally, the regulation of *musk* by Agrin via binding of GAPB to the N-box is similar if not identical to that proposed for the localization of *AChR $\epsilon$*  and  $\delta$  subunit gene expression to the synapse (Schaeffer et al., 1998). The promoters of *musk* and of *AChR $\epsilon$* , when activated by Agrin, respond in a similar way to interfering with Agrin-dependent signaling components, e.g., ErbBs (Meier et al., 1998b) or JNK, and their N-boxes compete for the same components of nuclear extracts. Thus, Agrin-activated MuSK sets up signaling loops that are not only used to control its own expression but also that of *AChR $\epsilon$*  (Fig. 7) and potentially of other genes regulated via an N-box, such as *utrophin* (Angus et al., 2001), *AChR $\delta$  subunit* (Koike et al., 1995; Duclert et al., 1996; Fromm and Burden, 1998; Schaeffer et al., 1998), and *AChE* genes (Gramolini et al., 1998; Chan et al., 1999). Induction of *musk* by agrin-activated MuSK and, via the same pathway, of major synaptic genes downstream of MuSK is a simple way for the nerve to orchestrate by the secretion of Agrin the formation of the postsynaptic membrane and to stabilize it when the synapse begins to transmit impulses.

## Materials and methods

### Screening of a mouse genomic library

The genomic library (packaged in Lambda FIX II Vector<sup>®</sup>; Stratagene) was screened with a dig-labeled 580-bp fragment corresponding to the 5' part of the mouse *nsk2*/MuSK cDNA (Ganju et al., 1995). Approximately  $3 \times 10^6$  plaques were screened, and two positive clones containing upstream and downstream regions from the first exon were further purified. 1.4 kb of upstream and 1 kb of downstream sequence, respectively, were subcloned into the pBluescript KS<sup>®</sup> vector (Stratagene) and sequenced by using the Dye-Deoxy Termination cycle sequencing method. The sequence data available from GenBank/EMBL/DBJ under accession no. AY114512.

### 5' RACE analysis

The transcription start site was determined by 5' RACE. RNA was extracted from C2C12 cells using the RNeasy Mini Kit (QIAGEN). 5' RACE was performed using the 5' RACE system version 2 (GIBCO BRL). The cDNA was amplified using the anchor primer and an internal primer R1, TCTTCAAC-CAAGGCATCTACAAG. The PCR products were cloned into pGEM-T vector<sup>®</sup> (Promega), and 20 independent clones were sequenced.

### Construction of the reporter constructs

**p1.6luc-N.** The 1.6-kb genomic fragment containing the region upstream of the first exon was amplified by PCR by using the oligonucleotides 1.6F, GAGAAGCTTCCCTTTCTTTGACTATAG and 1.6R, GAGAAGCTTACT-GTTTGTGTCTGCAGCGC. This PCR product was cloned into the HindIII site of the pGL3 vector containing the luciferase gene as a reporter (Promega) to give p1.6luc. The same strategy was used to insert downstream of the luciferase gene into the BamHI site of pGL3 a 1-kb fragment of the first intron of *nsk2/musk* containing the N-box by using the NboxFB, GAGGGATTCTCCAGAGTGTTTGTCTCTG and the NboxRB, GAGG-GATTCTCTAGAACTCTGCATAACGC oligonucleotides, giving p1.6luc-N.

**p1.6luc-Nmut.** Two mutations were introduced into the N-box at +871 (Fig. 1 A) of p1.6luc-N by PCR: 871C>T and 872C>T. A first amplification with, respectively, NboxFB, etsmutR, CTTAGGTGGTGTATTTCGAAT-CATTCCAAAGGCA and NboxFR and etsmutF, TGCCTTTGGAATGTAT-TGGAAATAACACCACCTAAG was performed. The two products were mixed, denatured for 5 min at 95°C, annealed 10 min at 56°C, and the an-

nealed product was submitted to a second PCR by using the NboxFB and NboxFR primers. After sequencing, the mutated fragment was cloned into the pGL3 vector as described above.

**p1.6nlslacZ-Nmut.** For some experiments, luciferase in p1.6luc-N was replaced by nlslacZ, a reporter construct directing  $\beta$ -Gal expression to nuclei for easier microscopic observation. For this purpose, a KpnI-NcoI fragment was excised from a 1.6luc construct and subcloned in a pLacF plasmid (Sanes et al., 1991). The fragment containing the N-box was added downstream the lacZ at the SphI restriction site, using the same primers as described above, but using SphI instead of the BamHI restriction site. The plasmid DNA was resuspended either in Tris (5 mM) for cell transfection and in vivo intracellular injection or in Tris 5 mM/NaCl 0.9%, pH, 7.4, for in vivo electroporation.

**Other constructs.** Other constructs used have been described as indicated: pnlGFP-S6 (Hashemolhosseini et al., 2000); pHER2, pHER2KM, pHER4, pHER4KM (Weiss et al., 1997); pGABP $\beta$ DN, pGABP $\alpha$ -myc, pGABP $\beta$ -myc (Briguet and Rüegg, 2000); pJNK-APF (Gupta et al., 1995), pMKK7D (Wang et al., 1998); pLCF216 $\epsilon$  (Jones et al., 1996); pRacV12, pRacN17 (Joneson et al., 1996).

### Cell culture and transfection

C2C12 cells were cultured according to Jones et al (1999).  $8 \times 10^4$  cells were passaged onto 6-well plates that had been coated with either laminin alone or with laminin and mini-Agrin (N25;c21B8) comprising the Nta laminin-binding site and the minimal c21AgrinB8 of chicken Agrin (Gesemann et al., 1996; Meier et al., 1998a). This mini-Agrin is sufficient to induce *AChRe* subunit gene expression in vivo. Unlike full-length Agrin, it does not carry glycosaminoglycan side chains and thus cannot bind and accumulate NRG-1 without signaling through MuSK (Jones et al., 1996; Meier et al., 1998a,b). Myoblasts were transfected with 800 ng of reporter plasmid, 80 ng of pRL-TK plasmid (Promega) for normalization, and 150 ng of the appropriate plasmid using 6  $\mu$ l of Eugene6 reagent (Roche Diagnostics). Myotubes were analyzed 7 d after transfection using the dual luciferase reporter assay system (Promega). Light intensity was quantified with a Turner Designs Model 20e Luminometer. NRG-1 was from R&D Systems.

### Nuclear extracts and electrophoretic mobility shift assays

Muscle nuclei were collected from differentiated C2C12 myotubes according to the protocol of Schreiber et al. (1989) with the exception that all buffers contained a cocktail of protease inhibitors (Roche Diagnostics).

For the mobility shift assay experiments, the oligonucleotides were as follows: etsF, TGGAAATGACCCGAAATAACACCA; etsR, TGGTGT-TATTCGGGTACATTCCA; etsmutF, TGGAAATGATTTGGAAATAACACCA; etsmutR, TGGTGTATTTCGAATACATTCCA;  $\epsilon$ compF, AAACCTA-GCCCGGAACCTAACACCC; and  $\epsilon$ compR, GGGTGTAGTTCCGGGCT-AGGTTT. Annealed oligonucleotide probes were labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. For the binding reaction, 2 ng of labeled probe, 2  $\mu$ g of poly dIdC, and 3–4  $\mu$ g of nuclear extract were mixed and incubated for 20 min at RT, loaded on a 5% polyacrylamide gel and run at 200 V for 40 min. The gel was dried and exposed in a Storm imaging system (Amersham Biosciences). Antibodies against GABP $\alpha$  and GABP $\beta$  used in supershift experiments have been characterized by Schaeffer et al. (1998).

### ErbB2 immunoprecipitation and Western blotting

C2C12 myotubes cultured in 10 cm plates were washed twice with ice-cold PBS, and membrane proteins were extracted with 1 ml detergent extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 1% Triton X-100, and 1 $\times$  protease inhibitor cocktail [Roche Diagnostics]). Extracts were cleared from cell debris by centrifugation at 4°C. For each sample, the similar amounts of total protein was subjected to ErbB2 immunoprecipitation with 0.1  $\mu$ g/ml anti-erbB2 antibody (C18; Santa Cruz Biotechnology, Inc.) for 1 h at 4°C and incubated with protein A–Agarose beads (Roche Diagnostics) overnight at 4°C. After washing five times with 1 ml detergent extraction buffer, excess liquid was removed, and the beads were boiled in 50  $\mu$ l SDS sample buffer. Samples were separated on a 5% acrylamide gel and transferred to Immobilon-P (Millipore) by electroblotting. ErbB2 protein was detected by a polyclonal rabbit anti-ErbB2 antibody (a gift from A. Badache, Friedrich Miescher Institute, Basel, Switzerland) and with peroxidase-conjugated anti-rabbit antibody. Immunocomplexes were visualized followed by ECL.

### Injections of plasmids into muscle fibers in vivo

Male Wistar rats were anaesthetized with Nembutal (1 ml/kg). Single fibers of rat soleus were injected in their proximal end plate-free region as de-

scribed (Jones et al., 1997) with 100 ng/ $\mu$ l of the reporter plasmid, 50 ng/ $\mu$ l of the pRL-TK plasmid, the plasmids indicated, and with 50 ng/ $\mu$ l of the pnlGFP-S6 plasmid (Jones et al., 1999) for later identification of injected fibers. After 2 wk, rats were killed with CO<sub>2</sub>, and the soleus muscles were excised for analysis using the Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega). Blockade of ectopic subsynaptic differentiation in vivo by JNK-APF was tested as described by Briguet and Rüegg (2000).

### Electroporation of muscles

Rats were anaesthetized, and the left soleus muscle was surgically exposed. 30  $\mu$ l of a DNA mixture containing 55  $\mu$ g of the p1.6luc-N or p1.6nlslacZ-N plasmids and 35  $\mu$ g of the pRL-TK plasmid were injected into the soleus with a Hamilton syringe (gauge 34). After suturing the surgical cut, two stainless steel plate electrodes, covered with a conductive gel allowing electrical contact with the skin, were placed at each side of the leg. Eight pulses of 20-ms duration were delivered at 1-s intervals from a T830 square wave electroporator (BTX) (Mir et al., 1999). Analysis of luciferase activities was as described above. Muscles electroporated with p1.6nlslacZ-N were stained for  $\beta$ -Gal activity and subsequently for acetylcholinesterase (Koelle and Friedenwald, 1949). Fiber bundles teased from the muscles were examined under the microscope at 400 $\times$ .

We thank Drs. L. Schaeffer and J.P. Changeux, (Institut Pasteur Paris, France), for GABP antibodies, and Dr. A. Briguet (Biozentrum, Basel, Switzerland) for GABP expression plasmids, Annick Werner for excellent technical assistance and Drs. B. Bettler, M.A. Rüegg and P. Escher for comments on the manuscript.

This work was supported by the Swiss National Science Foundation and the Swiss Foundation for Research on Muscle Diseases.

Submitted: 28 October 2002

Revised: 14 April 2003

Accepted: 14 April 2003

## References

- Alroy, I., L. Soussan, R. Seger, and Y. Yarden. 1999. Neu differentiation factor stimulates phosphorylation and activation of the Sp1 transcription factor. *Mol. Cell. Biol.* 19:1961–1972.
- Altiok, N., S. Altiok, and J.P. Changeux. 1997. Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. *EMBO J.* 16:717–725.
- Angus, L.M., R.Y. Chan, and B.J. Jasmin. 2001. Role of intronic E- and N-box motifs in the transcriptional induction of the acetylcholinesterase gene during myogenic differentiation. *J. Biol. Chem.* 276:17603–17609.
- Apel, E.D., D.J. Glass, L.M. Moscoso, G.D. Yancopoulos, and J.R. Sanes. 1997. Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron.* 18:623–635.
- Batchelor, A.H., D.E. Piper, F.C. de la Brousse, S.L. McKnight, and C. Wolberger. 1998. The structure of GABP $\alpha$ /beta: an ETS domain-ankyrin repeat heterodimer bound to DNA. *Science.* 279:1037–1041.
- Bowen, D.C., J.S. Park, S. Bodine, J.L. Stark, D.M. Valenzuela, T.N. Stitt, G.D. Yancopoulos, R.M. Lindsay, D.J. Glass, and P.S. DiStefano. 1998. Localization and regulation of MuSK at the neuromuscular junction. *Dev. Biol.* 199:309–319.
- Briguet, A., and M.A. Rüegg. 2000. The Ets transcription factor GABP is required for postsynaptic differentiation in vivo. *J. Neurosci.* 20:5989–5996.
- Burden, S., and Y. Yarden. 1997. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron.* 18:847–855.
- Chan, R.Y., C. Boudreau-Lariviere, L.M. Angus, F.A. Mankal, and B.J. Jasmin. 1999. An intronic enhancer containing an N-box motif is required for synapse- and tissue-specific expression of the acetylcholinesterase gene in skeletal muscle fibers. *Proc. Natl. Acad. Sci. USA.* 96:4627–4632.
- Cohen, N.A., W.E. Kaufmann, P.F. Worley, and F. Rupp. 1997. Expression of Agrin in the developing and adult rat brain. *Neuroscience.* 76:581–596.
- Davis, R.J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell.* 103:239–252.
- de Kerchove D'Exaerde, A., J.R. Cartaud, and L. Schaeffer. 2002. Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression. *EMBO Rep.* 3:1075–1081.
- Duclert, A., N. Savatier, and J.P. Changeux. 1993. An 83-nucleotide promoter of the acetylcholine receptor epsilon-subunit gene confers preferential synaptic

- expression in mouse muscle. *Proc. Natl. Acad. Sci. USA*. 90:3043–3047.
- Duclert, A., N. Savatier, L. Schaeffer, and J.P. Changeux. 1996. Identification of an element crucial for the sub-synaptic expression of the acetylcholine receptor epsilon-subunit gene. *J. Biol. Chem.* 271:17433–17438.
- Fischbach, G.D., and K.M. Rosen. 1997. ARIA: a neuromuscular junction neuregulin. *Annu. Rev. Neurosci.* 20:429–458.
- Fromm, L., and S.J. Burden. 1998. Synapse-specific and neuregulin-induced transcription require an ets site that binds GABPalpha/GABPbeta. *Genes Dev.* 12:3074–3083.
- Fromm, L., and S.J. Burden. 2001. Neuregulin-1-stimulated phosphorylation of GABP in skeletal muscle cells. *Biochemistry.* 40:5306–5312.
- Ganju, P., E. Walls, J. Brennan, and A.D. Reith. 1995. Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. *Oncogene.* 11:281–290.
- Gesemann, M., V. Cavalli, A.J. Denzer, A. Brancaccio, B. Schumacher, and M.A. Ruegg. 1996. Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron.* 16:755–767.
- Gramolini, A.O., E.A. Burton, J.M. Tinsley, M.J. Ferns, A. Cartaud, J. Cartaud, K.E. Davies, J.A. Lunde, and B.J. Jasmin. 1998. Muscle and neural isoforms of agrin increase utrophin expression in cultured myotubes via a transcriptional regulatory mechanism. *J. Biol. Chem.* 273:736–743.
- Gupta, S., D. Campbell, B. Derijard, and R.J. Davis. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science.* 267:389–393.
- Hashemolhosseini, S., C. Moore, L. Landmann, A. Sander, H. Schwarz, V. Witzemann, B. Sakmann, and H.R. Brenner. 2000. Electrical activity and postsynapse formation in adult muscle: gamma-AChRs are not required. *Mol. Cell. Neurosci.* 16:697–707.
- Hoffmeyer, A., A. Avots, E. Flory, C.K. Weber, E. Serfling, and U.R. Rapp. 1998. The GABP-responsive element of the interleukin-2 enhancer is regulated by JNK/SAPK-activating pathways in T lymphocytes. *J. Biol. Chem.* 273:10112–10119.
- Ip, F.C., D.G. Glass, D.R. Gies, J. Cheung, K.O. Lai, A.K. Fu, G.D. Yancopoulos, and N.Y. Ip. 2000. Cloning and characterization of muscle-specific kinase in chicken. *Mol. Cell. Neurosci.* 16:661–673.
- Jones, G., A. Herczeg, M.A. Ruegg, M. Lichtsteiner, S. Kroger, and H.R. Brenner. 1996. Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells. *Proc. Natl. Acad. Sci. USA*. 93:5985–5990.
- Jones, G., T. Meier, M. Lichtsteiner, V. Witzemann, B. Sakmann, and H.R. Brenner. 1997. Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proc. Natl. Acad. Sci. USA*. 94:2654–2659.
- Jones, G., C. Moore, S. Hashemolhosseini, and H.R. Brenner. 1999. Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. *J. Neurosci.* 19:3376–3383.
- Joneson, T., M. McDonough, D. Bar-Sagi, and L. Van Aelst. 1996. RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science.* 274:1374–1376.
- Koelle, G.B., and J.S. Friedenwald. 1949. A histochemical method for localizing cholinesterase activity. *Proc. Soc. Exp. Biol. Med.* 70:617–622.
- Koike, S., L. Schaeffer, and J.P. Changeux. 1995. Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor delta-subunit gene. *Proc. Natl. Acad. Sci. USA*. 92:10624–10628.
- Leu, M., E. Bellmunt, M. Schwander, I. Farinas, H.R. Brenner, and U. Muller. 2003. Erbb2 regulates neuromuscular synapse formation and is essential for muscle spindle development. *Development.* 130:2291–2301.
- Lin, W., R.W. Burgess, B. Dominguez, S.L. Pfaff, J.R. Sanes, and K.F. Lee. 2001. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature.* 410:1057–1064.
- Loeb, J.A., T.S. Khurana, J.T. Robbins, A.G. Yee, and G.D. Fischbach. 1999. Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. *Development.* 126:781–791.
- Luo, Z., Q. Wang, J. Zhou, J. Wang, M. Liu, X. He, A. Wynshaw-Boris, W. Xiong, B. Lu, and L. Mei. 2002. Regulation of AChR clustering by dishevelled interacting with MuSK and PAK1. *Neuron.* 35:489–505.
- Meier, T., D.M. Hauser, M. Chiquet, L. Landmann, M.A. Ruegg, and H.R. Brenner. 1997. Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J. Neurosci.* 17:6534–6544.
- Meier, T., P.A. Marangi, J. Moll, D.M. Hauser, H.R. Brenner, and M.A. Ruegg. 1998a. A minigene of neural agrin encoding the laminin-binding and acetylcholine receptor-aggregating domains is sufficient to induce postsynaptic differentiation in muscle fibres. *Eur. J. Neurosci.* 10:3141–3152.
- Meier, T., F. Masciulli, C. Moore, F. Schoumacher, U. Eppenberger, A.J. Denzer, G. Jones, and H.R. Brenner. 1998b. Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. *J. Cell Biol.* 141:715–726.
- Mir, L.M., M.F. Bureau, J. Gehl, R. Rangara, D. Rouy, J.M. Caillaud, P. Delaere, D. Branellec, B. Schwartz, and D. Scherman. 1999. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA*. 96:4262–4267.
- Moore, C., M. Leu, U. Muller, and H.R. Brenner. 2001. Induction of multiple signaling loops by MuSK during neuromuscular synapse formation. *Proc. Natl. Acad. Sci. USA*. 98:14655–14660.
- Moscoso, L.M., G.C. Chu, M. Gautam, P.G. Noakes, J.P. Merlie, and J.R. Sanes. 1995. Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/hergulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev. Biol.* 172:158–169.
- Ruegg, M.A., K.W. Tsim, S.E. Horton, S. Kroger, G. Escher, E.M. Gensch, and U.J. McMahan. 1992. The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron.* 8:691–699.
- Sandrock, A.W., Jr., S.E. Dryer, K.M. Rosen, S.N. Gozani, R. Kramer, L.E. Theill, and G.D. Fischbach. 1997. Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. *Science.* 276:599–603.
- Sanes, J.R., Y.R. Johnson, P.T. Kotzbauer, J. Mudd, T. Hanley, J.C. Martinou, and J.P. Merlie. 1991. Selective expression of an acetylcholine receptor-lacZ transgene in synaptic nuclei of adult muscle fibers. *Development.* 113:1181–1191.
- Sapru, M.K. 2001. Neuregulin-1 regulates expression of the Ets-2 transcription factor. *Life Sci.* 69:2663–2674.
- Schaeffer, L., N. Duclert, M. Huchet-Dymanus, and J.P. Changeux. 1998. Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. *EMBO J.* 17:3078–3090.
- Schaeffer, L., A. de Kerchove D'Exaerde, and J.P. Changeux. 2001. Targeting transcription to the neuromuscular synapse. *Neuron.* 31:15–22.
- Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
- Si, J., Q. Wang, and L. Mei. 1999. Essential roles of c-JUN and c-JUN N-terminal kinase (JNK) in neuregulin-increased expression of the acetylcholine receptor epsilon-subunit. *J. Neurosci.* 19:8498–8508.
- Tang, J., S.A. Jo, and S.J. Burden. 1994. Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle. *Development.* 120:1799–1804.
- Tansey, M.G., G.C. Chu, and J.P. Merlie. 1996. ARIA/HRG regulates AChR epsilon subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway. *J. Cell Biol.* 134:465–476.
- Valenzuela, D.M., T.N. Stitt, P.S. DiStefano, E. Rojas, K. Mattsson, D.L. Compton, L. Nunez, J.S. Park, J.L. Stark, D.R. Gies, et al. 1995. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron.* 15:573–584.
- Wang, Y., B. Su, V.P. Sah, J.H. Brown, J. Han, and K.R. Chien. 1998. Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. *J. Biol. Chem.* 273:5423–5426.
- Weiss, F.U., C. Wallasch, M. Campiglio, W. Issing, and A. Ullrich. 1997. Distinct characteristics of heregulin signals mediated by HER3 or HER4. *J. Cell. Physiol.* 173:187–195.
- Weston, C., B. Yee, E. Hod, and J. Prives. 2000. Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. *J. Cell Biol.* 150:205–212.
- Yang, X., S. Arber, C. William, L. Li, Y. Tanabe, T.M. Jessell, C. Birchmeier, and S.J. Burden. 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron.* 30:399–410.