The Erk MAP Kinase Pathway Is Activated at Muscle Spindles and Is Required for Induction of the Muscle Spindle-Specific Gene Egr3 by Neuregulin

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Muscle spindles are sensory receptors composed of specialized muscle fibers, known as intrafusal muscle fibers, along with the endings of sensory neuron axons that innervate these muscle fibers. Formation of muscle spindles requires neuregulin1 (NRG1), which is released by sensory axons, activating ErbB receptors in muscle cells that are contacted. The transcription factor Egr3 is transcriptionally induced by NRG1, which in turn activates various target genes involved in forming intrafusal fibers. We have previously shown that, in cultured muscle cells, NRG1 signaling activates the Egr3 gene through SRF and CREB, which bind to a composite regulatory element, and that NRG1 signaling targets SRF by stimulating nuclear translocation of SRF coactivators myocardin-related transcription factor (MRTF)-A and MRTF-B and targets CREB by phosphorylation. The current studies examined signaling relays that might function in the NRG1 pathway upstream of SRF and CREB. We found that transcriptional induction of Egr3 in response to NRG1 requires the MAP kinase Erk1/2, which acts upstream of CREB to induce its phosphorylation. MRTFs are targeted by the Rho-actin pathway, yet in the absence of Rho-actin signaling, even though MRTFs fail to be translocated to the nucleus, NRG1 induces Egr3 transcription. In mouse muscle in vivo, activation of Erk1/2 is enhanced selectively where muscle spindles are located. These results suggest that Erk1/2 acts in intrafusal fibers of muscle spindles to induce transcription of Egr3 and that Egr3 induction occurs independently of MRTFs and involves Erk1/2 acting on other transcriptional regulatory targets that interact with the SRF-CREB regulatory element.

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Muscle spindles are sensory receptors embedded within muscle that detect changes in muscle length. Muscle spindles convey length information to the CNS through their associated sensory neurons. This information is processed in the brain to help sense positions of limbs and other body parts. Muscle spindles also participate in stretch reflexes, which involves the direct action of their innervating sensory neurons on motor neurons to resist muscle stretch.

Each muscle spindle is composed of specialized muscle fibers, known as intrafusal muscle fibers, along with the endings of axons from proprioceptive sensory neurons that innervate these fibers. Within the spindle, several intrafusal fibers are arranged in parallel with other muscle fibers and are encapsulated by connective tissue. These fibers have distinctive characteristics, including their morphology and gene expression pattern, which includes having distinctive isoforms of myosin heavy chain (Pedrosa-Domellof and Thornell, 1994). Intrafusal muscle fibers are innervated by axons, also known as afferent fibers, of sensory neurons. The endings of these fibers spiral around and terminate on central portions of intrafusal fibers and contain stretch-sensitive ion channels for performing sensory function. Because intrafusal muscle fibers are embedded in parallel with other muscle fibers, the length of intrafusal fibers changes in conjunction with the entire muscle.

Formation of muscle spindles involves reciprocal interactions between developing muscle fibers and proprioceptive sensory afferents that contact them (Maier, 1997). Specification of a muscle fiber to develop into an intrafusal fiber of a muscle spindle requires inductive interactions of sensory afferent endings with developing muscle fibers (Kucera et al., 1993). Indeed, in mice lacking sensory neurons as a result of loss of signaling by

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particular neurotrophic factors, muscle spindles fail to develop (Emfors et al., 1994; Klein et al., 1994).

According to mouse genetic approaches, the NRG1-ErbB signaling pathway is a critical component of the inductive interactions between sensory axons and muscle. NRG1, a secreted signaling protein, is produced by various neurons, including proprioceptive sensory neurons, and released from axons. In mice in which NRG1 is inactivated in a variety of neurons, including sensory neurons that innervate muscle spindles, muscle spindles fail to develop even though sensory neurons form normally and contact muscle fibers (Hippenmeyer et al., 2002). ErbB2, which is a key component of the NRG1 receptor, is present in muscle fibers. Likewise, in mice lacking ErbB2 in skeletal muscle, muscle spindles fail to form (Andrechek et al., 2002; Leu et al., 2003). Furthermore, in cultured muscle cells, NRG1 stimulates expression of various genes associated with intrafusal fibers, including the characteristic myosin heavy chain isoforms (Jacobson et al., 2004). Activated ErbB receptors likely are directly linked to ShcA, a docking protein that interacts directly with activated receptor tyrosine kinases, including ErbB receptors, based on mouse genetic studies showing that spindle formation requires ShcA in skeletal muscle (Hardy et al., 2007). Several specific domains of ShcA were found to be required, including those that interact with activated receptors and one that interacts with the adaptor Grb2, suggesting that, in developing spindles, signaling by activated ErbB receptors is linked to recruitment of ShcA, which in turn binds to Grb2. Based on these mouse genetic studies, when a proprioceptive sensory axon contacts a developing muscle fiber, NRG1 that is released by the axon activates ErbB receptors in the muscle fiber that is contacted. Activation of ErbB receptors, likely through ShcA and Grb2 to which they are linked, then initiates an intracellular signaling pathway that ultimately contributes to that muscle fiber forming into an intrafusal fiber of a muscle spindle.

A critical component of the intracellular pathway that acts downstream of ErbBs is the transcription factor Egr3, which is transcriptionally induced as part of NRG1-ErbB signaling and in turn activates various target genes involved in formation of muscle spindles. Egr3 is a member of a family of transcription factors whose original member was identified as an immediate early gene that is induced upon growth factor stimulation (O’Donovan et al., 1999). Expression of Egr3 is induced in developing intrafusal fibers following innervation by sensory axons but is not detected in extrafusal fibers, which do not receive sensory innervation (Tourtellotte and Milbrandt, 1998). Induction of Egr3 occurs as part of the NRG1-ErbB pathway, because Egr3 expression is induced in cultured primary human myotubes following stimulation with NRG1 (Jacobson et al., 2004) and in mice in which either NRG1 or ErbB2 is disrupted, Egr3 induction in muscle fails to occur (Hippenmeyer et al., 2002; Leu et al., 2003). Egr3 is required to form muscle spindles because, in mice lacking Egr3, muscle spindles fail to develop (Tourtellotte and Milbrandt, 1998). Furthermore, forced expression of Egr3 throughout the muscle induces expression of intrafusal fiber-specific genes and converts all muscle fibers to intrafusal fibers (Albert et al., 2005). Thus, NRG1 acts, at least in part, by inducing transcription of Egr3, which in turn activates various target genes involved in formation of muscle spindles.

The signaling machinery that connects ErbBs to Egr3 transcriptional induction is presumably critical for muscle spindle formation. Previously we examined transcriptional regulatory elements and associated proteins that function during NRG1-ErbB signaling. We found, using cultured muscle cells, that NRG1 signaling activates the Egr3 gene through SRF and CREB, which bind to a composite regulatory element, and that NRG1 signaling targets SRF, by stimulating nuclear translocation of SRF coactivators myocardin-related transcription factor (MRTF)-A and MRTF-B and targets CREB through phosphorylation (Herndon et al., 2013; Herndon and Fromm, unpublished experiments). The current studies examine signaling relays that might function in the NRG1 pathway upstream of SRF and CREB. We found that induction of Egr3 by NRG1 requires the Erk1/2 MAP kinase pathway, which targets CREB, and that the Erk1/2 MAP kinase pathway is activated selectively at muscle spindles in vivo. We found that MRTFs are targeted by the Rho-actin pathway but that Erg3 induction occurs independently of this pathway, suggesting that induction of Erg3 in response to NRG1 involves Erk1/2 acting on other transcriptional regulatory targets that interact with the SRF-CREB regulatory element.

MATERIALS AND METHODS

Cell Culture

C2C12 myoblasts were propagated using growth media (DMEM, 20% bovine growth serum [Hyclone, Logan, UT], 50 μg/ml gentamycin). To induce differentiation into myotubes, cells were grown to confluence, and the medium was replaced with differentiation media (DMEM, 4% horse serum, 50 μg/ml gentamycin).

qRT-PCR

C2C12 myotubes that had been in differentiation for 4 days were serum starved (except for myotubes that would be treated with NRG1 for 24 hr) for 2 hr and either stimulated with NRG1 (HRGB1; R&D Systems, Minneapolis, MN; 100 ng/ml) or left untreated, and, after the indicated time, RNA was isolated from cells using RNA Stat-60 (Tel-Test). For 24-hr NRG1 treatment, C2C12 myotubes that had been in differentiation medium for 4 days cells were placed in differentiation medium containing 1% horse serum either with or without NRG1, and 24 hr later RNA was isolated from cells. Inhibitors were added to myotubes prior to NRG1 stimulation: U0126 (Cell Signaling Technology, Beverly, MA; 20 μM for 30 min), Clostridium toxin B (EMD Chemicals, Billerica, MA; 50 ng/ml for 2 hr), latrunculin B (EMD Chemicals; 0.5 μM for 55 min), wortmannin (Cell Signaling Technology; 10 μM for 1 hr), and cyclosporin A (EMD Chemicals; 5 μM for 30 min); inhibitors remained throughout NRG1 treatment. Myotubes in the
Fig. 1. NRG1 induces rapid and sustained induction of Egr3 expression in myotubes. C2C12 myotubes were treated with NRG1 for the indicated time or left untreated (−), and the level of Egr3 mRNA was measured. For each time point, the amount of Egr3 mRNA, after normalizing to actin mRNA, is shown relative to the untreated sample (mean expression ± SEM; n = 2 per time point).

Western Blotting

C2C12 myotubes that had been in differentiation media for 4 days were serum starved for 2 hr and either stimulated with NRG1 for 10 min or left untreated. U0126 (20 μM) was added 30 min prior to NRG-1 stimulation and remained throughout NRG-1 treatment. Myotubes were lysed and proteins from cell extracts were analyzed by Western blotting as described by Herndon et al. (2013) using antibodies (all from Cell Signaling Technology) to phosphorylated Erk1/2 (No. 4370; diluted 1:2,000), total Erk1/2 (No. 4695; diluted 1:1,000), serine 133-phosphorylated CREB (No. 4276; diluted 1:1,000), or total CREB (No. 9197; diluted 1:1,000), followed by peroxidase-conjugated secondary antibody.

Immunofluorescence Staining

C2C12 myotubes that had been in differentiation media for 4 days were serum starved for 2 hr and stimulated with NRG1 for either 10 min (for pErk1/2 and MRTF staining) or 4 hr (for Egr3 staining) or left untreated. Inhibitors were added to myotubes prior to NRG1 stimulation: U0126 (20 μM for 30 min), Clostridium toxin B (50 ng/ml for 2 hr), and latrunculin B (0.5 μM, 25 min); inhibitors remained throughout NRG1 treatment. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked for 30 min with 1% bovine serum albumin (for MRTF staining) or 5% normal goat serum (for pErk1/2 and Egr3 staining) in PBS. Cells were probed with antibodies to MRTF-A (Santa Cruz Biotechnolog, Santa Cruz, CA; sc-21558; diluted 1:100) or MRTF-B (Santa Cruz Biotechnolog; sc-47282; diluted 1:100), or normal IgG (2 μg/ml) for 1 hr at room temperature or with antibodies to phosphorylated Erk1/2 (Cell Signaling Technology; No. 4370; diluted 1:200) or Egr3 (Santa Cruz Biotechnolog; sc-191; diluted 1:50) overnight at 4°C, followed by Alexa Fluor 555-conjugated secondary antibody (Life Technologies, Grand Island, NY; A21428 or A21432; each diluted 1:500) for 30–60 min and counterstained with 300 nM DAPI for 5 min.

YFP-thy1 transgenic mice (Feng et al., 2000) at P0 were perfused with 4% paraformaldehyde in PBS. Hindlimbs were dissected, placed in 30% sucrose in PBS overnight at 4°C, embedded and frozen in OCT medium, and sectioned longitudinally at 12 μm with a cryostat onto gelatin-coated slides. Sections were permeabilized with 0.3% Triton X-100 in PBS for 15 min and blocked with 5% normal goat serum in PBS for 30 min. Sections were probed with an antibody to phosphorylated Erk1/2 (Cell Signaling Technology; No. 4370), diluted 1:200 in blocking solution, overnight at 4°C, followed by Alexa Fluor 555 anti-rabbit IgG (Life Technologies; No. A21428) diluted 1:500 in blocking solution, overnight at 4°C, followed by Alexa Fluor 488 anti-mouse IgG (Life Technologies; No. A21432) diluted 1:500 in blocking solution for 1 hr, and counterstained with 300 nM DAPI in PBS for 5 min. Stained cells and sections were visualized with a LSM 5 Pascal confocal microscope (Zeiss).

RESULTS

NRG1 induces Egr3 expression in nascent intrafusal muscle fibers and in cultured primary myotubes. C2C12 cells, an established, fusion-competent, muscle cell line, are a simple source of cells and might provide a good system for studying the signaling pathway by which NRG1 induces Egr3. We examined the transcriptional response of Egr3 to NRG1 in C2C12 cells. As shown in Figure 1, NRG1 induces expression of Egr3 in C2C12 myotubes. The level of Egr3 mRNA increases robustly within 1 hr, the earliest time point examined, and remains elevated, although at slightly reduced levels, for at least 24 hr, the latest time point examined. We also examined changes in Egr3 protein levels in response to NRG1, using immunofluorescence cell staining. As shown in Figure 2E,F, in myotubes treated with NRG1, a robust increase in Egr3 staining in nuclei was seen compared with myotubes without NRG1. The increase in Egr3 staining from NRG1 stimulation is found almost entirely in fused, multinucleated cells morphologically identifiable as myotubes rather than the mononucleated cells resembling myoblasts that were also present in the culture. Thus, C2C12 cells appear to be a useful system for studies on how Egr3 is induced in myotubes by NRG-ErbB signaling. Furthermore, the rapid and sustained induction of Egr3 in these cells is consistent with a role in coordinating expression of downstream genes specific for intrafusal muscle fiber structure and function.
The MAP kinase cascade involving the Erk1/2 class of MAP kinases commonly functions downstream of receptor tyrosine kinases, including ErbB receptors, and can act on transcription factors to activate gene expression. We determined the role of the Erk1/2 pathway during NRG1 signaling using the inhibitor U0126, which...
blocks activation of Erk1/2 by inhibiting its upstream activating kinase, MEK. We initially examined Erk1/2 activation in response to NRG1 by Western blotting and by immunofluorescence cell staining using an antibody that recognizes only the active form of Erk1/2 that is dually phosphorylated in its activation loop by MEK. As shown by Western blotting in Figure 2A, both Erk1 and Erk2 are activated in myotubes in response to NRG1, whereas, in cells treated with U0126, Erk1/2 activation is blocked. Immunofluorescence also confirms Erk1/2 activation in NRG1-stimulated cells that is blocked by U0126 (Fig. 2B,C). Activated Erk1/2 detected by immunofluorescence is present only in the cytoplasm and is not translocated to the nucleus and is found almost entirely in fused, multinucleated cells morphologically identifiable as myotubes rather than the mononucleated cells resembling myoblasts that were also present in the culture. We then evaluated expression of the Egr3 gene in response to NRG1 in myotubes in which Erk activation was blocked by treatment with U0126. As shown in Figure 2, in myotubes treated with U0126, induction of the Egr3 gene in response to NRG1, as determined by measuring mRNA levels, was almost completely abolished (Fig. 2D), and induction of Egr3 protein, as determined by immunofluorescence cell staining, was also substantially impaired (Fig. 2G,H), indicating that induction of Egr3 by NRG1 requires Erk1/2. The Erk1/2 pathway often activates gene regulatory proteins that induce transcription of target genes. To determine whether Erk1/2 signaling activates Egr3 at the transcriptional level, we performed a transcriptional reporter assay using a gene fusion consisting of an hGH reporter linked to 2.2 kb of upstream flanking sequence from the Egr3 gene, which we had previously shown was responsive to NRG1. We transfected C2C12 muscle cells with the Egr3–hGH gene fusion and measured the amount of hGH expression from myotubes in which Erk activation was blocked by treatment with U0126. As shown in Figure 2I, treatment with U0126 completely blocked NRG1 responsiveness of the transcriptional reporter, indicating that the Erk1/2 pathway induces Egr3 at the transcriptional level, presumably by acting on transcription factors that target the Egr3 gene.

According to our previous studies, NRG1 signaling activates the Egr3 gene through SRF and CREB. Furthermore, NRG1 signaling targets SRF by stimulating nuclear translocation of SRF coactivators MRTF-A and MRTF-B and targets CREB through phosphorylation. The Erk1/2 pathway can act upstream of CREB by phosphorylating and activating Msk-type kinases that in turn directly phosphorylate CREB at serine 133 (Johannessen et al., 2004). To determine whether the Erk1/2 pathway acts upstream of CREB during NRG1 signaling in myotubes, we examined CREB phosphorylation in NRG-1-stimulated myotubes in which Erk activation was blocked by treatment with U0126. To examine CREB phosphorylation in these cells, we performed Western blotting with an antibody specific to serine 133-phosphorylated CREB. As shown in Figure 3A, in myotubes treated with U0126, induction of CREB phosphorylation in response to NRG1 was blocked, indicating that Erk1/2 functions upstream of CREB during NRG1 signaling.

Although the mechanism by which NRG1 signaling stimulates translocation of MRTFs to the nucleus has not been previously reported, the predominant pathway by which MRTFs are translocated to the nucleus in response to certain other extracellular signals is through activation of Rho GTPases and the resulting actin filament polymerization (Olson and Nordheim, 2010). In addition, Erk1/2, by directly phosphorylating MRTFs, can at least in some instances regulate MRTF function, including by affecting MRTF nuclear localization (Kalita et al., 2006; Muehlich et al., 2008). To examine the roles of both the Rho-actin and the Erk1/2 pathways in regulating MRTF nuclear localization during NRG1 signaling in myotubes, we performed immunofluorescence staining of MRTFs in the presence of inhibitors for each pathway. We had previously found by immunofluorescence that MRTFs in myotubes without NRG1 were largely localized to the cytoplasm, with little to no MRTFs detected in nuclei, whereas, in myotubes stimulated with NRG1, MRTFs were localized predominantly to nuclei. The cells in which MRTFs were detected consisted mainly of fused, multinucleated cells morphologically identifiable as myotubes and generally not the mononucleated cells resembling myoblasts that were also present in the culture. As shown in Figure 3B–E, blocking Erk1/2 activation by treatment with U0126 did not have any effect on MRTF nuclear localization in response to NRG1, indicating that pathways other than Erk1/2 control MRTF nuclear localization.

In the Rho-actin signaling relay, which can stimulate MRTF nuclear localization in response to certain extracellular signals, Rho GTPases are activated through Rho guanine nucleotide exchange factors that interact with activated cell-surface receptors. Rho GTPases, through several effectors, then stimulate assembly of globular G actin subunits into filamentous F actin polymers. In the nonpolymerized state, G actin interacts with MRTFs, causing MRTFs to be retained in or exported to the cytoplasm. The reduction in G actin concentration resulting from Rho-mediated assembly into F actin allows MRTFs to enter the nucleus. To examine the role of the Rho-actin pathway, we interfered with it at two different steps. We used Clostridium toxin B, which catalyzes glucosylation of Rho-family proteins (Just et al., 1995) and inhibits their interaction with effector proteins (Sehr et al., 1998). We also used the natural compound latrunculin B, which binds G-actin and inhibits its polymerization into F actin and its dissociation with MRTFs (Miralles et al., 2003). As shown in Figure 4A–J, although NRG1 normally stimulates MRTF translocation to the nucleus, treatment with either toxin B or latrunculin B results in MRTFs being retained in or exported to the cytoplasm, with little to none detected in nuclei, which is the same pattern of MRTF localization observed in unstimulated myotubes. Thus, NRG1 signaling in myotubes stimulates MRTF nuclear localization through a pathway involving activation of Rho GTPases and the resulting actin filament polymerization.
Given that the Rho-actin pathway targets the SRF coactivators MRTF-A and MRTF-B and that SRF and its binding site are involved in induction of Egr3 transcription by NRG1, we asked whether induction of the Egr3 gene by NRG1 depends on the Rho-actin pathway. We evaluated expression of the Egr3 gene in response to NRG1 in myotubes in which the Rho-actin pathway had been blocked by treatment with either toxin B or latrunculin B. As shown in Figure 4K, in myotubes treated with either of these compounds, transcriptional induction of Egr3 by NRG1 occurs normally. Thus, induction of Egr3 transcription during NRG1 signaling occurs independently of the Rho-actin pathway and the resulting translocation of MRTFs to the nucleus. We also examined the roles of signaling through two other pathways, phosphatidylinositol 3 (PI3) kinase, which is commonly linked to receptor tyrosine kinases, and calcineurin, which is involved in NRG1-stimulated activation of the Egr-family member Egr2 in differentiating Schwann cells (Kao et al., 2009). We inhibited PI3K with wortmannin and calcineurin with cyclosporin A in myotubes. As shown in Figure 5A, in myotubes treated with either wortmannin or calcineurin, transcriptional induction of Egr3 by NRG1 occurs normally, indicating that induction of Egr3 transcription occurs independently of PI3 kinase and calcineurin signaling.

Because activation of the Erk1/2 MAP kinase pathway but not other pathways that we examined is required for transcriptional induction of Egr3 by NRG1, the Erk1/2 pathway in particular might be activated in intrafusal fibers of muscle spindles when Egr3 is being induced (see Fig. 5B for pathway model). To determine whether induction of Egr3 expression at muscle spindles coincides with activation of Erk1/2, we evaluated activation of Erk1/2 in muscle of mice by immunofluorescence using an antibody that recognizes only the active form of Erk1/2 that is dually phosphorylated in its activation loop by MEK. We used hindlimb muscle from newborn (P0) mice, a stage in which NRG1 signaling in intrafusal fibers is expected to be active, because Egr3 expression is first detectable in nascent intrafusal fibers at about E15, when proprioceptive sensory afferents make initial contacts, and remains expressed until at least P10 (Tourtellotte et al., 2001). To facilitate identification of muscle spindles, we used transgenic mice in which yellow fluorescent protein (YFP), under the control of the neuron-specific thy1 promoter, is expressed throughout neurons (Feng et al., 2000), and we located spindles in longitudinal muscle sections based on the characteristic annulospiral appearance of nerve fibers. As shown in Figure 6, phosphorylated Erk1/2 that is dually phosphorylated in its activation loop by MEK. We used hindlimb muscle from newborn (P0) mice, a stage in which NRG1 signaling in intrafusal fibers is expected to be active, because Egr3 expression is first detectable in nascent intrafusal fibers at about E15, when proprioceptive sensory afferents make initial contacts, and remains expressed until at least P10 (Tourtellotte et al., 2001). To facilitate identification of muscle spindles, we used transgenic mice in which yellow fluorescent protein (YFP), under the control of the neuron-specific thy1 promoter, is expressed throughout neurons (Feng et al., 2000), and we located spindles in longitudinal muscle sections based on the characteristic annulospiral appearance of nerve fibers. As shown in Figure 6, phosphorylated Erk1/2, which reflects Erk activation, is enhanced selectively where muscle spindles are located. Phosphorylated Erk1/2 at spindles appears to be present within muscle fibers rather than within endings of axons from proprioceptive sensory neurons, because phosphorylated Erk1/2 staining and YFP-containing axons generally do not overlap. The ring-like staining pattern for phosphorylated Erk1/2 resembles the staining pattern for proteins located at the plasma membrane, although definitive subcellular
localization of phosphorylated Erk1/2 within muscle fibers has not been determined. Outside of muscle spindles, phosphorylated Erk1/2 was mostly undetectable, although it was found sporadically in no obvious pattern in nonspindle locations, whereas, in all muscle spindles that we examined, Erk1/2 activation was detected, suggesting that Erk1/2 is activated by signals that act on intrafusal fibers of muscle spindles.

**DISCUSSION**

Our results showing that Erk1/2 is activated at muscle spindles and is required for Egr3 transcriptional induction by NRG1 suggest that Erk1/2 functions in muscle spindle formation. Mouse genetic studies showing a requirement in muscle spindle formation for ShcA, including the domain that interacts with the adaptor protein Grb2 (Hardy et al., 2007), are also consistent with a role of Erk1/2, because Grb2, after binding to ShcA that is docked on activated receptor tyrosine kinase, activates the Erk1/2 MAP kinase pathway by interacting with the Sos guanine nucleotide exchange factor for Ras (Ravichandran, 2001). Our results further suggest that a critical function of Erk1/2 in intrafusal fibers of muscle spindles is to induce transcription of Egr3 in these cells. Erk1/2 presumably induces Egr3 during NRG1 signaling by directly or indirectly targeting transcriptional regulatory proteins that regulate expression of the Egr3 gene. Based on our

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**Fig. 4.** Transcriptional induction of Egr3 during NRG1 signaling occurs independently of the Rho-actin pathway and the resulting translocation of MRTFs to the nucleus. A-J: Blocking the Rho-actin pathway with latrunculin B (latB) or with toxin B (toxB) prevents NRG1-stimulated translocation of MRTF-A and MRTF-B from the cytoplasm to the nucleus. Myotubes without inhibitor (A,B,G,H) or in the presence of the inhibitors latB (C,D,I,J) or toxB (E,F) that had been unstimulated (A,C,E,G,I) or stimulated with NRG1 (B,D,F,H,J) were fixed, permeabilized, and stained by immunofluorescence with antibodies to MRTF-A (A–F) and MRTF-B (G–J). K: C2C12 myotubes were treated with NRG1 in the absence or presence of the Rho-actin pathway inhibitors toxB or latB, and the level of Egr3 mRNA was measured. For each condition, the amount of Egr3 mRNA after normalizing to actin mRNA is shown relative to without NRG1 or inhibitors (mean expression ± SEM). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
previous studies suggesting that the transcriptional activities of CREB and SRF are activated during NRG1 signaling, the Erk1/2 MAP kinase pathway might target CREB, SRF, or their interacting proteins.

The Erk1/2 MAP kinase pathway is known to activate CREB by phosphorylating and activating Msk-type kinases that in turn directly phosphorylate CREB at serine 133. Phosphorylation of CREB at serine 133, along with other events, facilitates recruitment of the CREB coactivators CBP and p300, thereby enhancing transcription (Johannessen et al., 2004). Because we found that NRG1-induced phosphorylation of CREB in myotubes requires activation of Erk1/2, CREB is likely being phosphorylated through the action of Erk1/2 on Msk kinases. The functional relevance for CREB phosphorylation in induction of the Egr3 gene in response to NRG1 is suggested by our previous studies (Herndon et al., 2013). According to these studies, which used muscle cells in culture, a regulatory element that contains a CREB-binding site functions as an NRG1 response element, and CREB binds to the regulatory region of the Egr3 gene that contains this element. Furthermore, through CREB inactivation, we showed directly that CREB is involved in Egr3 transcriptional induction by NRG1. To induce CREB-mediated transcription, phosphorylation of CREB is widely accepted to be a key event, although additional cooperating events that vary by stimulus are required (Johannessen et al., 2004). Thus, the finding that, in response to NRG1, CREB is phosphorylated and functions to induce Egr3 transcription strongly suggests that targeting CREB through phosphorylation is one of the events required for inducing Egr3 transcription. In vivo, we found that, even though activation of Erk1/2 is enhanced selectively where muscle spindles are located, CREB is activated widely in muscle fibers located throughout the muscle (data not shown). CREB, besides being phosphorylated by Msk kinases, can be phosphorylated by a variety of other kinases that are activated in response to many different types of signals, so presumably signals are present outside of muscle spindles that activate CREB independently of Erk1/2. Although induction of Egr3 transcription in intrafusal fibers, in which Egr3 expression is restricted, might involve stimulation of CREB phosphorylation, which is suggested by muscle cell culture studies, the widespread CREB activation that we observed in muscle in vivo suggests that targeting CREB is not sufficient to activate Egr3 in intrafusal fibers. Additional transcription factor targets could thus be involved, possibly including SRF and its interacting proteins, which would be consistent with the known requirement for additional events, in cooperation with CREB phosphorylation, that activate CREB-mediated transcription (Ravnskjaer et al., 2007).

We showed in previous studies that NRG1 signaling targets SRF by stimulating nuclear translocation of SRF coactivators MRTF-A and MRTF-B. In the current studies, we show that MRTFs are translocated through the action of the Rho–actin pathway, yet, when the Rho–actin pathway is inhibited, NRG1 nevertheless

**Fig. 5.** A: Transcriptional induction of Egr3 during NRG1 signaling occurs independently of the PI3 kinase and calcineurin pathways. C2C12 myotubes were treated with NRG1 in the absence or presence of Wortmannin (WM) or cyclosporin A (CsA), and the level of Egr3 mRNA was measured. For each condition, the amount of Egr3 mRNA after normalizing to actin mRNA was shown relative to that without NRG1 or inhibitors (mean expression ± SEM). B: Model for signaling pathway that induces formation of intrafusal muscle fibers. Components in boldface have been demonstrated via mouse genetic approaches. Components that are underscored have been demonstrated in this study by using muscle cells in culture. For components in italics, their roles in this pathway have not been directly examined but are presumed to function based on their presence within canonical receptor tyrosine kinase signaling pathways.
induces Egr3 transcription normally. Because, when the Rho-actin pathway is inhibited in NRG1-stimulated myotubes, MRTFs in nuclei are almost entirely undetectable even though Egr3 transcription is induced normally, induction of Egr3 in response to NRG1 presumably occurs independently of MRTFs. Our previous results demonstrating a role of SRF and its binding site in NRG1 responsiveness of the Egr3 gene nevertheless suggest a role of SRF in NRG1 signaling, though based on our current studies one in which SRF is regulated independent of both MRTFs and the Rho–actin pathway.

In addition to the myocardin/MRTF family, SRF interacts with other proteins that might regulate its ability to activate transcription in response to NRG1. Another family of SRF coactivators that is well known for having a signal-regulated function is the p62TCF family, which comprises the Ets proteins Elk1, Elk3, and Elk4. These coactivators are activated through phosphorylation by several kinases, including by Erk1/2 (Treisman, 1994). The p62TCF family is not likely to be involved in Egr3 transcriptional induction, however, because interaction of p62TCF with SRF requires DNA binding of p62TCF to an Ets site adjacent to the SRF site, and, for the functional SRF that we identified in the Egr3 transcriptional regulatory region, no nearby potential Ets binding sites are recognizable. In addition to the two well-characterized families of signal-regulated SRF coactivators, myocardin/MRTF and p62TCF, SRF is known to interact with various other coactivators that enhance its ability to activate transcription, including members of the GATA family (Morin et al., 2001), certain homeodomain family proteins (Phiel et al., 2001; Zhang et al., 2008), and several widely used transcriptional coactivators, including CBP/p300, activating signal coactivator, and steroid receptor coactivator (Ramirez et al., 1997; Kim et al., 1998; Jung et al., 2002; Hanna et al., 2009), raising the possibility of other SRF cofactors functioning in NRG1 responsiveness. In support of the involvement of other SRF cofactors, CBP/p300 can be phosphorylated, including by Erk1/2, affecting its binding to transcription factors and transcriptional activity (Serra et al., 2007; Hanna et al., 2009; Xia et al., 2011). In addition, the ability of SRF to interact with CBP/p300 might suggest a mechanism of cooperativity with CREB, which occupies a binding site in the Egr3 transcriptional regulatory region that is adjacent to the SRF site, in facilitating recruitment of CBP/p300 to phosphorylated CREB. Future studies will address whether transcriptional induction of Egr3 by NRG1 involves the Erk1/2 MAP kinase pathway targeting particular SRF-interacting proteins or whether additional transcriptional regulatory proteins are targeted that interact with other NRG1-responsive regulatory elements.

Our studies pointing to a role of the Erk1/2 pathway in activating Egr3 transcription by NRG1 in cultured myotubes suggest that the Erk1/2 pathway might function in developing muscle spindles by inducing expression of Egr3 in intrafusal muscle fibers. In nascent intrafusal fibers, activation of Egr3 by NRG1 begins during embryonic development.

Fig. 6. In mouse muscle in vivo, activation of Erk1/2 is enhanced selectively where muscle spindles are located. Longitudinal sections of hindlimb muscle at P0 from thy1-YFP transgenic mice, which express YFP throughout neurons, were stained by immunofluorescence with antibodies to phospho-Erk1/2 (A,C,E,G). Spindles (arrowheads) were located based on the characteristic annulospiral appearance of YFP-containing nerve fibers (B,D,F,H). Two representative sections that contain muscle spindles (A–D and E–H) are shown, each at a lower magnification (A,B,E,F) and at a higher magnification (C,D,G,H), centered on a spindle (in E,F, which contain two spindles, only the upper spindle is shown at higher magnification in G,H, and the orientation of this section is somewhat skewed from longitudinal). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
development, when proprioceptive sensory afferents that release NRG1 contact certain myotubes. Our studies in which NRG1 is added to cultured C2C12 myotubes were designed to model this developmental process, but it is not clear precisely how well the cultured myotubes that we used mimic the embryonic myotubes that are being induced by NRG1 in vivo. C2C12 myotubes certainly have many molecular and morphological similarities to myotubes in vivo, but it is nonetheless possible that the molecular mechanisms by which NRG1 induces Egr3 transcription differs between myotubes of the C2C12 line and those being induced by NRG1 during development. Future in vivo studies might determine whether Erk1/2 and its potential downstream targets actually function in developing muscle spindles.

The ring-like staining pattern for activated Erk1/2 at muscle spindles resembles the staining pattern for proteins located at the plasma membrane. The subcellular locations in which activated Erk1/2 can be found depend on its interactions with particular proteins that control its compartmentalization, which ultimately determines substrates that Erk1/2 phosphorylates. Erk1/2 can be brought to the plasma membrane based on its interaction with scaffolding proteins that organize Ras, Mek, and Erk into a signaling complex. Within such a signaling complex, Raf, in cells in which Ras is activated in response to a particular stimulus, would be recruited to the plasma membrane by interacting with Ras, resulting in the Raf, Mek, and Erk components becoming sequentially activated (Kolch, 2005). Erk1/2 that becomes activated often dissociates from its scaffold and acts in additional subcellular locations. At muscle spindles, even if the primary location for activated Erk1/2 is the plasma membrane, because Erk1/2 often remains active for only a limited time following exposure of cells to a stimulating signal, activated Erk1/2 might nevertheless dissociate and act transiently in other subcellular locations, with activated Erk1/2 found at the plasma membrane representing the population that was most recently activated. In myotubes in culture that are stimulated with NRG1, in which Erk1/2 is activated in a largely synchronous fashion, activated Erk1/2 is found throughout the cytoplasm, suggesting the Erk1/2 might be directed to cytoplasmic substrates in intrafusal muscle fibers. The subcellular locations in which Erk1/2 might function will be addressed in future studies on the targets of the Erk1/2 MAP kinase pathway that are critical in inducing Egr3 transcription.

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REFERENCES


