A Role for Neuregulin1 Signaling in Muscle Spindle Differentiation

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Summary

The maturation of synaptic structures depends on inductive interactions between axons and their prospective targets. One example of such an interaction is the influence of proprioceptive sensory axons on the differentiation of muscle spindles. We have monitored the expression of three transcription factors, Egr3, Pea3, and Erm, that delineate early muscle spindle development in an assay of muscle spindle-inducing signals. We provide genetic evidence that Neuregulin1 (Nrg1) is required for proprioceptive afferent-evoked induction of muscle spindle differentiation in the mouse. Ig-Nrg1 isoforms are preferentially expressed by proprioceptive sensory neurons and are sufficient to induce muscle spindle differentiation in vivo, whereas CRD-Nrg1 isoforms are broadly expressed in sensory and motor neurons but are not required for muscle spindle induction.

Introduction

During the development of the nervous system, the differentiation and maturation of neuronal target cells is frequently triggered by signals supplied by ingrowing axons. Such inductive interactions can control the specification of prospective target cells (Huang and Kunes, 1998; Huang et al., 1998) or promote the maturation of prespecified targets (Lin et al., 2001; Yang et al., 2001; Arber et al., 2002). Analysis of the differentiation of mechanoreceptive organs associated with the peripheral terminals of vertebrate primary sensory neurons has provided clear examples of the inductive influence of neurons on target cells (Zelena, 1994). Mechanoreceptors are responsible for the initial detection of mechanical sensory stimuli, transducing mechanical to electrical stimuli through specialized contacts with sensory terminals. Among vertebrate sensory transduction systems, the specialized endings formed by the peripheral terminals of group Ia proprioceptive afferents with muscle spindles have been well characterized at a structural and functional level (Hunt, 1990; Zelena, 1994). Muscle spindles—stretch-sensitive mechanoreceptors that lie in parallel with skeletal muscle fibers—comprise a “fusiform” capsule that contains distinct intrafusal muscle fiber types that can be classified by their stereotypic arrangement of nuclei (Hunt, 1990; Zelena, 1994; Maier, 1997). The differentiation of muscle spindles in rodents begins during embryonic development (Kozeka and Ontell, 1981; Kucera and Walro, 1994; Zelena, 1994), and their maturation continues well into postnatal life (Zelena, 1994).

The contribution of neural inputs to the initiation and maintenance of muscle spindle differentiation has traditionally been analyzed by surgical manipulation at early postnatal stages (Kucera and Walro, 1992; Kucera et al., 1993; Zelena, 1994). After elimination of sensory input, muscle spindles rapidly degenerate (Kucera et al., 1993), suggesting a critical requirement for sensory innervation early in muscle spindle differentiation. This view has been strengthened by the failure of muscle spindle differentiation in mice mutant for the genes encoding the neurotrophin NT-3 or its receptor TrkC, mutants in which proprioceptive neurons fail to differentiate (Ernfors et al., 1994; Klein et al., 1994; Farinas et al., 1994; Liebl et al., 1997). Intrafusal fibers within muscle spindles are also innervated by motor neurons (Zelena, 1994), but similar surgical manipulations have failed to reveal a role for motor axons in the initial differentiation of muscle spindles (Kucera and Walro, 1992). Together, these studies support the idea that proprioceptive afferents are a selective source of inductive signals required to induce the differentiation of muscle spindles from immature myofibers (Ernfors, 2001; Farinas, 1999). The identity of the postulated afferent-derived factor(s) responsible for the induction of muscle spindle differentiation is unclear.

Intrafusal muscle fibers within developing muscle spindles have been shown to express several transcription factors. Egr3, a transcription factor of the zinc-finger class (Tourtellotte and Milbrandt, 1998; O’Donovan et al., 1999), and Pea3 and Erb1, two transcription factors of the ETS family (Sharrocks, 2001), are each expressed by intrafusal but not extrafusal muscle fibers (Arber et al., 2000), and so provide molecular markers with which to probe early steps of muscle spindle differentiation. The identification of extrinsic signals responsible for the induction of ETS and Egr3 expression may provide clues...
to the identity of afferent-derived spindle inductive factor(s). One notable feature of the expression of Pea3, Erb1, and Egr3 in certain cell types is their activation by a Neuregulin-1 (Nrg1)-triggered signaling cascade (O'Hagan and Hassell, 1998; Bosc et al., 2001; Shepherd et al., 2001; Sweeney et al., 2001), raising the possibility that Nrg1 signaling might be involved in early steps of muscle spindle differentiation.

The Nrg1 gene is subject to differential promoter usage and alternative splicing, resulting in the expression of distinct transmembrane and secreted Nrg1 protein isoforms. Each isoform contains an EGF-like motif, and this domain is essential for all known Nrg1 biological activities (Garratt et al., 2000; Buonanno and Fischbach, 2001). Two major classes of Nrg1 proteins can be distinguished on the basis of their domain architecture. One class, characterized by an extracellular cysteine-rich domain (CRD), has been termed the CRD-Nrg1 (or Type III Nrg1) isoform (Meyer et al., 1997; Garratt et al., 2001; Wolpowitz et al., 2000; Buonanno and Fischbach, 2001). The second class, containing an extracellular immunoglobulin (Ig)-like domain in the absence of a CRD domain, has been termed the Ig-Nrg1 isoform and includes Type I and Type II structures (Fischbach and Rosen, 1997; Meyer et al., 1997; Garratt et al., 2001; Buonanno and Fischbach, 2001). All of the known Nrg1 protein isoforms signal through the activation of heterodimeric transmembrane tyrosine kinase receptors of the ErbB class (Burden and Yarden, 1997).

Both CRD-Nrg1 and Ig-Nrg1 isoforms have been proposed to influence the differentiation of postsynaptic skeletal muscle fibers at the neuromuscular junction (Buonanno and Fischbach, 2001; Schaeffer et al., 2001). In vitro studies have provided evidence that Nrg1 activity triggers a signaling cascade that results in the activation of ETS proteins which, in turn, leads to the synapse-specific transcription of genes encoding acetylcholine receptor subunits and other muscle proteins (Schaeffer et al., 1998; Fromm and Burden, 1998; Briguet and Ruegg, 2000; Buonanno and Fischbach, 2001). The expression of the CRD-Nrg1 isoform has also been implicated in the differentiation and survival of Schwann cells in the peripheral nervous system as well as in the maintenance of synaptic inputs from motor neuron to muscle (Wolpowitz et al., 2000). Although the CRD-containing Nrg1 isoforms are widely expressed by DRG and motor neurons (Meyer et al., 1997; Yang et al., 1998; Loeb et al., 1999), expression of transcripts encoding Ig domain-containing isoforms has been reported to exhibit a more restricted pattern of expression in developing dorsal root ganglion (DRG) neurons (Meyer et al., 1997). The functional roles of Ig domain-containing isoforms expressed by sensory neurons are unclear, since mouse mutants lacking these isoforms die from cardiac defects at early embryonic stages (Meyer and Birchmeier, 1995; Kramer et al., 1996).

In this study, we have used the early expression of the transcription factors Egr3, Pea3, and a Pea3-related ETS transcription factor Erm by intrafusal fibers within muscle spindles to test the potential role of Nrg1 as an inducer of muscle spindle differentiation in the mouse. We show that Ig-Nrg1 isoforms are expressed preferentially by TrkC+ DRG sensory neurons at a developmental stage when proprioceptive afferents first invade muscles, whereas only a very low level of Ig-Nrg1 is expressed in developing motor neurons. In contrast, CRD-Nrg1 is expressed broadly by most, or all, DRG neurons and motor neurons. We have compared the state of muscle spindle differentiation in two different mouse Nrg1 mutants. Elimination of all Nrg1 isoforms from DRG and motor neurons profoundly impairs muscle spindle differentiation, as assessed by the absence of Egr3, Pea3, and Erm expression, and results in the failure of proprioceptive afferents to elaborate annulospiral terminals. In contrast, muscle spindle differentiation proceeds normally in mice that selectively lack CRD-Nrg1 isoforms. These assays reveal a critical role for Nrg1 signaling in the early induction of muscle spindle differentiation and establish the sufficiency of Ig-Nrg1 isoforms in this inductive process.

Results

Transcription Factor Expression by Embryonic Muscle Spindles

To define early markers of muscle spindle differentiation, we focused on the expression of genes encoding two classes of transcription factors, ETS and Egr proteins, members of which are expressed by intrafusal muscle fibers (Tourtellotte and Milbrandt, 1998; Arber et al., 2000). We found that three transcription factors, Egr3 and the ETS genes Pea3 and Erm, are expressed by intrafusal muscle fibers at early stages of muscle spindle differentiation in the developing hindlimb. Expression of Egr3, Pea3, and Erm in muscle spindles was first detected at E15.5, and expression persisted at least up to P10 (Figures 1A–1I; data not shown). From E15.5 to at least E18.5, expression of Erm, but not of Pea3 or Egr3, was also detected in intrafusal muscle fibers (Figures 1C, 1F, 1L, and 1J), in a domain that appeared to coincide with the synaptic endplate band, as assessed by the localization of GAP-43+ axonal terminals and the position of high-density clusters of acetylcholine receptors labeled by α-bungarotoxin (BTX) binding (Figures 1K and 1L). Together, the selectivity of expression of these genes permits an early molecular distinction between intrafusal and extrafusal muscle fibers.

The early expression of Egr3, Pea3, and Erm by intrafusal fibers in differentiating muscle spindles raises the issue of whether these genes are regulated independently or in an interrelated manner. To assess this, we analyzed the expression of these genes in muscle spindles of Pea3 and Egr3 mouse mutants (Tourtellotte and Milbrandt, 1998; Livet et al., 2002). Pea3 and Erm expression were unaffected in Egr3 mutants (S.H., S.A., W.G. Tourtellotte, and T.M.J., unpublished observation). Similarly, expression of Egr3 and Erm by muscle spindles is normal in Pea3 mutant mice (unpublished observation; Livet et al., 2002). The early embryonic lethality of Erm mutant mice precluded analysis of muscle spindle development (S.A., J.A. Hassell, and T.M.J., unpublished observation). Nevertheless, these findings suggest that Egr3, Pea3, and Erm provide independent genetic markers of early stages of muscle spindle differentiation.

Proprioceptive Innervation of Nascent Muscle Spindles

Proprioceptive afferents have been implicated in the induction of muscle spindle differentiation (Zelena, 1994;
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Figure 1. Transcription Factor Expression by Embryonic Muscle Spindles

(A–I) Time course of Egr3 (A, D, and G), Pea3 (B, E, and H), and Erm (C, F, and I) expression by intrafusal muscle fibers of hindlimb muscles of wild-type embryos at E15.5 (A–C), E16.5 (D–F), and E18.5 (G–I).

(J) Expression of Erm by myonuclei located within the synaptic endplate band at E18.5.

(K and L) Double-label immunocytochemistry on adjacent section to (J) to reveal clusters of AChRs with α-bungarotoxin (BTX: K, white and L, green) and motor axons with GAP-43 (L, red). Note that expression of Erm in the synaptic endplate band is also present in (C), (F), and (I) in addition to its expression in intrafusal muscle fibers.

Scale bar equals 40 μm in (A)–(I) and 80 μm in (J)–(L).

Maier, 1997; Ernfors, 2001). We therefore asked whether the onset of Egr3, Pea3, and Erm expression in muscle spindles is regulated by the ingrowth of proprioceptive axons in hindlimb muscles. To assess the development of proprioceptive afferent endings, we analyzed the expression of the calcium binding protein Parvalbumin (PV), a selective marker of proprioceptive afferent neurons (Honda, 1995; Arber et al., 2000).

PV+ axons were first detected within developing hindlimb muscles between E15 and E15.5 (Figures 2A–2C). At this stage, PV+ axons had made initial contacts with myofibers and had elaborated rudimentary terminals in a region marked by Egr3+ nuclei (Figures 2A–2C). From E15.5 to E16.5, PV+ axons branched at the central domain of nascent intrafusal muscle fibers (Figures 2D and 2E; Arber et al., 2000). From E16.5 to P3, proprioceptive axons developed more elaborate annulospiral endings around the central domain of intrafusal muscle fibers (Figures 2F and 2G). Over the period from E15.5 to P3, a second set of PV+ axons is located near myotendinous junctions, at the site of differentiating Golgi Tendon Organs (Figures 2H, 2I, and 2K). Thus, initial contacts between PV+ proprioceptive afferents and prospective intrafusal muscle fibers precede or coincide with the onset of expression of Egr3, Pea3, and Erm. In turn, the onset of expression of these transcription factors by intrafusal muscle fibers precedes the elaborate terminal branching of proprioceptive afferents.

To determine whether the presence of proprioceptive afferent fibers is required for the induction of Pea3, Egr3, and Erm expression in nascent muscle spindles, we took advantage of the fact that proprioceptive sensory neurons are lost at early developmental stages in TrkC mutant embryos (Klein et al., 1994; Liebl et al., 1997). An analysis of the expression of Pea3, Egr3, and Erm in E15.5 TrkC mutants revealed a lack of PV+ peripheral axon terminals and the absence of Pea3, Egr3, or Erm expression in intrafusal muscle fibers of hindlimb muscles (data not shown), consistent with reports of the lack of mature muscle spindles in these mutants (Farinaznas, 1999; Matsuo et al., 2000). Thus, signals provided by proprioceptive afferents are required to induce the expression of early transcriptional markers of intrafusal muscle fiber differentiation.

Selective Expression of Ig-Nrg1 Isoforms in Proprioceptive Sensory Neurons

To begin to define the molecular basis of the proprioceptive afferent-induced expression of Egr3, Pea3, and Erm in intrafusal muscle fibers, we focused on the signaling factor Nrg1, a gene known to be expressed by DRG and motor neurons (Meyer et al., 1997; Yang et al., 1998; Loeb et al., 1999; Garratt et al., 2000) and to induce ETS and Egr3 gene expression in other cellular contexts (O’Hagan and Hassell, 1998; Bosc et al., 2001; Shepherd et al., 2001; Sweeney et al., 2001; Parkinson et al., 2002). We analyzed the expression pattern of two major isoforms of Nrg1—one containing an Ig domain and the other containing a CRD domain—by in situ hybridization.
Figure 2. Proprioceptive Afferent Morphology in Developing Hindlimb Muscles

Proprioceptive afferent terminal elaboration at nascent muscle spindles (A–H) or GTOs (H–L) visualized by the expression of PV (white: A, B, D–F, H, and I–L) or double-label immunocytochemistry to PV (green) and Egr3 (red, C and G) in hindlimb muscles of E15.5 (A–C), E16.5 (D, E, I, and J), E18.5 (H, K, and L), and P3 (F and G) wild-type (A–I and K) and Isl1Cre/Nrg1flox/H11002 mutant (J and L) mice.

(H) Low-magnification view of PV^+ proprioceptive afferents in an E18.5 gracilis muscle innervating nascent muscle spindles located in the central domain of the muscle (red arrows) and prospective GTOs (yellow arrows) at the myotendinous junction (marked by dotted line).

Scale bar equals 20 μm in (A) and (I)–(L); 25 μm in (B)–(E); 40 μm in (F) and (G); and 80 μm in (H).

using isoform-specific probes (Meyer et al., 1997; Wolpowitz et al., 2000).

At E12.5, the CRD-Nrg1 isoform was expressed by most or all DRG neurons, and this expression pattern persisted until at least E18.5 (Figures 3C, 3F, 3I, and 3L). In contrast, expression of the Ig-Nrg1 isoform was first detected at E14.5, and between E14.5 and E18.5 it was restricted to a subpopulation of DRG neurons (Figures 3B, 3E, 3H, and 3K). These findings are consistent with previous studies on the differential pattern of expression of type I and type III isoforms of Nrg1 (Meyer et al., 1997). To determine whether DRG neurons that express the Ig-Nrg1 isoform are proprioceptive or cutaneous, we analyzed the expression of Ig-Nrg1 in the DRG of TrkC mutants (Klein et al., 1994; Liebl et al., 1997). We found that Ig-Nrg1 expression was absent in DRG neurons of TrkC mutants, analyzed at E17.5 (Figures 3K and 3N). We also analyzed neurogenin-1 (ngn-1) mutants in which a dramatic loss in TrkA^+ cutaneous DRG neurons and a preferential enrichment of proprioceptive neurons is observed (Ma et al., 1999). A marked increase in the density of Ig-Nrg1^+ neurons in the DRG of ngn-1 mutants was detected at E17.5 (Figures 3K and 3Q), supporting the idea that the Ig-Nrg1 isoform is expressed by proprioceptive neurons. We also analyzed CRD-Nrg1 expression in DRG neurons of TrkC and ngn-1 mutants. Expression of the CRD-Nrg1 isoform persisted in DRG neurons in TrkC and ngn-1 mutant embryos (Figures 3O and 3R). Together, these findings provide evidence that proprioceptive afferents coexpress the CRD-Nrg1 and Ig-Nrg1 isoforms of Nrg1, whereas cutaneous DRG neurons appear to express the CRD-Nrg1, but not the Ig-Nrg1 isoform.

Proprioceptive sensory, but not motor, neurons have been reported to induce muscle spindle differentiation (Maier, 1997), prompting us to compare Nrg1 isoform expression in embryonic motor neurons. CRD-Nrg1 isoforms were detected in motor neurons from E12.5 to E18.5 (Figure 3I; data not shown), consistent with previous findings (Meyer et al., 1997; Yang et al., 1998; Loeb et al., 1999). These findings establish that Ig-Nrg1 isoforms are expressed preferentially in proprioceptive neurons, whereas the CRD-Nrg1 isoform is expressed by motor as well as by sensory neurons.

To determine whether intrafusal muscle fibers are competent to respond to Nrg1-mediated signals, we analyzed expression of ErbB receptor subunits at late embryonic and early postnatal stages. At E18.5 to P1, ErbB3 expression was detected at neuromuscular junctions and in association with muscle spindles innervated...
Figure 3. Ig-Nrg1 but not CRD-Nrg1 Expression Is Restricted to Proprioceptive Afferents

Expression of TrkC (A, D, G, J, M, and P), Ig-Nrg1 (B, E, H, K, N, and Q), and CRD-Nrg1 (C, F, I, L, O, and R) in lumbar DRG of wild-type (A–L), TrkC−/− (M–O), and Ngn1−/− (P–R) embryos at E12.5 (A–C), E14.5 (D–F), and E17.5 (G–R).

(G–I) Low-magnification view of DRG including ventral horn of the spinal cord (marked by dotted line). CRD-Nrg1 (I) is expressed by motor neurons (arrows), whereas Ig-Nrg1 (H) and TrkC (G) expression is confined to DRG sensory neurons.

Scale bar equals 60 μm in (A)–(F) and (P)–(R); 140 μm in (G)–(I); and 80 μm in (J)–(O).

by PV+ proprioceptive nerve endings (Figures 4A–4F). We also detected weak expression of ErbB4 at nascent muscle spindles (data not shown). In contrast, we were not able to detect ErbB2 protein at embryonic or early postnatal stages, consistent with previous reports on ErbB localization at developing neuromuscular junctions (Zhu et al., 1995).

An Early Defect in Muscle Spindle Differentiation in Mice Lacking Nrg1 in DRG and Motor Neurons

To determine whether Nrg1 expression by DRG neurons is involved in the initiation of muscle spindle differentiation, we made use of a conditional Nrg1 allele in which the EGF-like motif present in all Nrg1 isoforms is flanked by loxP sites (Nrg1flox; Yang et al., 2001). To eliminate all Nrg1 isoforms from embryonic DRG and motor neurons, we used Isl1-directed expression of Cre-recombinase (Srinivas et al., 2001; Yang et al., 2001; see also Experimental Procedures). We first analyzed whether the expression of Egr3, Pea3, and Erm is initiated in intrafusal muscle fibers in Isl1Cre/Nrg1flox−/− mutants. The survival and initial differentiation of proprioceptive afferent sensory neurons is not impaired in Isl1Cre/Nrg1flox−/− mutants (see Supplemental Data and Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/36/6/1035/DC1), permitting us to identify the position of prospective intrafusal muscle fibers by their proximity to PV+ afferent endings at E16.5 and to analyze the expression of Egr3, Pea3, and Erm on adjacent sections. In hindlimb muscles of wild-type embryos, we identified Egr3+, Pea3+, and Erm+ muscle spindles in >60% of sections in which adjacent sections showed branched PV+ axons (Figures 5A–5D), and by E18.5, >90% of myofibers on sections adjacent to those containing PV+ terminals expressed Egr3, Pea3, and Erm (Figures 5I–5L). Thus, individual intrafusal muscle fibers are represented consistently in adjacent sections. In contrast, in Isl1Cre/Nrg1flox−/− mutants at E16.5 and E18.5, none of the myofibers contacted by PV+ axons expressed Egr3, Pea3, and Erm (Figures 5E–5H, 5M–5P, and 5S). However, expression of Erm in the synaptic endplate band of extrafusal muscle fibers contacted by α-motor neurons persisted in Isl1Cre/Nrg1flox−/− mutants (Figures 5E–5H, 5M–5P, and 5S). We also detected a lack of accumulation of ErbB3 in myofibers contacted by PV+ fibers in these
Figure 4. ErbB3 Receptor Expression at Nascent Muscle Spindles

Hindlimb muscles of P1 (A–C) and E18.5 (D–I; quadriceps) wild-type (A–F) and Isl1Cre/Nrg1flox/ mutant mice (G–I) analyzed by immunocytochemical staining of ErbB3 (white in A, D, and G; green in C; red in F and I), AChRs using α-bungarotoxin (BTX; white in B; red in C), and PV (white in E and H; green in F and I). Arrows mark nascent muscle spindle.

Scale bar equals 30 μm in (A)–(C) and 15 μm in (D)–(I).

mutants (Figures 4G–4I), consistent with the absence of expression of genes expressed selectively by intrafusal muscle fibers in Isl1Cre/Nrg1flox/ mutants.

We next examined whether the loss of muscle spindle differentiation in Isl1Cre/Nrg1flox/ mutants influences the morphology of PV+ proprioceptive afferent terminals. In E16.5 hindlimb muscles of Isl1Cre/Nrg1flox/ mutants, we found that PV+ proprioceptive afferents were present in normal numbers and initiated contact with individual myofibers, but these afferent fibers did not develop annulospiral branches around the myofibers (Figures 6A and 6E). In Isl1Cre/Nrg1flox/ mutants analyzed at E16.5, the morphology of PV+ proprioceptive afferents adjacent to muscle spindles resembled that found in wild-type muscle spindles at E15.5 (Figures 2A–2C and 6E). To determine whether the innervation of intrafusal muscle fibers in Isl1Cre/Nrg1flox/ mutants is simply delayed, we analyzed the innervation of muscle spindles at E18.5. In wild-type mice at this stage, PV+ proprioceptive terminals at muscle spindles have become elaborate (Figure 6B), but in Isl1Cre/Nrg1flox/ mutants, PV+ proprioceptive endings remained primitive and unbranched (Figure 6F). A similar defect in elaboration of terminal endings was evident when proprioceptive endings were visualized by GAP-43 expression (Figures 6C, 6D, 6G, and 6H). In contrast, PV+ afferents located at myotendinous regions that prefigure the position of Golgi Tendon Organs (Zelenka, 1994) possessed a flame-shaped arborization pattern similar to that in wild-type mice, when assayed at E16.5 to E18.5 (Figures 2I–2L).

Previous studies have shown that neuronally derived Nrg1 is essential for the survival of Schwann cells (reviewed by Garratt et al., 2000), raising the issue of whether the defects in muscle spindle differentiation in Isl1Cre/Nrg1flox/ mutants reflects a direct action of Nrg1 on myofibers or an indirect consequence of a perturbation in Schwann cell differentiation. To resolve this issue, we therefore examined the status of Schwann cell development in hindlimb muscles of Isl1Cre/Nrg1flox/ mutants.

We found that some hindlimb muscles exhibited complete elimination of S100+ Schwann cells at E16.5, whereas other muscles contained Schwann cells at wild-type numbers (Figures 7A, 7B, 7D, 7E, 7G, and 7H; data not shown; see Experimental Procedures for a likely explanation of the difference in Schwann cell survival to muscle spindles have become elaborate (Figure 6B), but in Isl1Cre/Nrg1flox/ mutants, PV+ proprioceptive endings remained primitive and unbranched (Figure 6F). A similar defect in elaboration of terminal endings was evident when proprioceptive endings were visualized by GAP-43 expression (Figures 6C, 6D, 6G, and 6H). In contrast, PV+ afferents located at myotendinous regions that prefigure the position of Golgi Tendon Organs (Zelenka, 1994) possessed a flame-shaped arborization pattern similar to that in wild-type mice, when assayed at E16.5 to E18.5 (Figures 2I–2L).

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CRD-Nrg1 Mutant Mice Do Not Exhibit Early Defects in Muscle Spindle Differentiation

We next examined whether CRD-Nrg1 isoforms are required for the induction of early muscle spindle differen-
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Figure 5. Lack of Induction of Egr3, Pea3, and Erm Expression in Intrafusal Muscle Fibers of Isl1Cre/Nrg1flox/Mutant Mice

(A–P) Analysis of muscle spindle differentiation at E16.5 (A–H) and E18.5 (I–P) in wild-type (A–D and I–L) and Isl1Cre/Nrg1flox/mutant (E–H and M–P) mice. Arrows point to the same muscle spindles on adjacent sections and asterisks (E–H) depict location of synaptic endplate band.
(A, E, I, and M) Immunocytochemical staining of PV (red) and AChRs using α-bungarotoxin (BTX; green).
(B, F, J, and N) In situ hybridization analysis of Erm expression.
(C, G, K, and O) In situ hybridization analysis of Egr3 expression.
(D, H, L, and P) In situ hybridization analysis of Pea3 expression.
(Q and R) Summary diagram of an embryonic muscle spindle in wild-type (Q) and Isl1Cre/Nrg1flox/mutant (R) mice. In wild-type (Q), nascent muscle spindles are innervated by annulospiral PV proprioceptive afferents and can be marked by the expression of Egr3, Pea3, and Erm. In Isl1Cre/Nrg1flox/mutant (R) mice, myotubes lack expression of Egr3, Pea3, and Erm and are innervated by unbranched proprioceptive afferents.
(S) Quantitation of percentage of muscle spindles in Isl1Cre/Nrg1flox/mutant mice relative to wild-type at E16.5 and E18.5. Numbers are based on the analysis of sections through the entire hindlimb of at least three independent embryos at each developmental stage. Analysis of individual muscles of the same embryos showed the same quantitative differences (data not shown). Scale bar equals 35 μm.

Blockade of Muscle Spindle Differentiation Does Not Influence the Central Projection Pattern of Proprioceptive Afferents

In the absence of Egr3, Pea3, and Erm expression by developing muscle spindles, the associated peripheral endings of PV+ proprioceptive afferents exhibit defects in the elaboration of annulospiral endings. This finding raises the issue of whether the development of the central terminal arbor of proprioceptive afferents might also be affected by the failure of muscle spindle differentiation. To assess this, we mapped the central projections of proprioceptive afferents in Isl1Cre/Nrg1flox/mutant mice at E16.5 and E18.5. No defects in the pattern of projections of PV+ afferents into the spinal cord, or in the extent of terminal arborization of PV+ axons in the ventral horn of the spinal cord, was detected in Isl1Cre/Nrg1flox/mutant mice (Figures 9A–9D). Thus, the blockade of the early steps in muscle spindle differentiation does not impair the pattern of central projections of proprioceptive afferents in the spinal cord.

Discussion

Muscle spindles are complex mechanoreceptors that provide sensory information critical for proprioception and the maintenance of muscle tone. Many classical studies have provided evidence that the differentiation of muscle spindles is initiated by signals supplied by the peripheral terminals of proprioceptive sensory neurons as they form intimate contacts with myofibers. In this study we provide evidence that Nrg1 proteins expressed by proprioceptive afferents are required for the
initiation of muscle spindle differentiation (Figure 10). Our findings show that Igga-Nrg1 isoforms supplied by proprioceptive afferents are sufficient to induce expression of the transcription factors Egr3, Pea3, and Erm in intrafusal muscle fibers, and thus to establish an early molecular distinction between intra- and extrafusal muscle fibers. The absence of Nrg1 expression also results in impaired branching of the peripheral terminals of group Ia proprioceptive afferents, presumably a secondary consequence of the absence of intrafusal muscle fibers. We discuss these findings in the context of: (1) the early expression and function of transcription factors induced in nascent intrafusal muscle fibers; (2) the role of Nrg1 isoforms in skeletal muscle fiber differentiation; (3) the role of target cell differentiation in the control of sensory axon terminal differentiation.

Muscle Spindle-Specific Expression of Transcription Factors

Intrafusal and extrafusal muscle fibers function in a profoundly different manner (Hunt, 1990; Zelena, 1994). Moreover, during embryonic development intrafusal fibers express several genes implicated in muscle function at strikingly higher levels than in extrafusal muscle fibers. Such genes include AChR subunit ε (Sanes et al., 1991), myosin isoforms (Walro and Kucera, 1999), and neurotrophic factors (Copray and Brouwer, 1994). Nevertheless, the precise time at which the properties of these two skeletal muscle fiber types diverge had not been clearly established, in part because of the lack of distinctive early molecular markers. The identification of transcription factors, notably Egr3 (Tourtellotte and Milbrandt, 1998) and Pea3 (Arber et al., 2000), which are expressed preferentially by intrafusal muscle fibers, reveals an early molecular divergence of these two muscle fiber types.

Genetic experiments in the mouse have explored the potential role of these transcription factors in muscle spindle differentiation. Egr3 mutant mice exhibit a sensory ataxia that appears to reflect a postnatal degeneration of muscle spindles, but the initiation of muscle spindle development is not compromised (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Pea3 is expressed by intrafusal fibers at early stages of muscle spindle differentiation, but Pea3 mutant mice do not show an obvious defect in muscle spindle differentiation (Livet et al., 2002; S.A. and T.M.J., unpublished observations), perhaps because of the coexpression of the closely related Erm gene. An additional Pea3 family member, Er81, is also expressed selectively by intrafusal muscle fibers (Arber et al., 2000). Er81 mutant mice exhibit a marked late onset (E18.5) degeneration of muscle spindles in a subset of limb muscles (Arber et al., 2000; Kucera et al., 2002), but it is unclear whether this defect results solely from the loss of Er81 from intrafusal fibers, or is a consequence of defects in proprioceptive sensory neurons, which also express this ETS factor (Lin et al., 1998; Arber et al., 2000). Thus, the analysis of mutant mice has not yet resolved the identity of the transcription factors that initiate the early cell-intrinsic steps of mus-
Neuregulin-1 and Muscle Spindle Differentiation

Figure 7. Schwann Cell Defects in Hindlimb Muscles of Isl1Cre/Nrg1flox/H11002 and CRD-Nrg1/H11002 Mutant Mice
Double-label immunocytochemistry of S100^{10} Schwann cells (red) and clusters of AChRs with α-bungarotoxin (BTX; green) in quadriceps (A–C), adductor (D–F), and gracilis (G–I) muscles of E16.5 hindlimbs of wild-type (A, D, and G), Isl1Cre/Nrg1flox/H11002 mutant (B, E, and H), and CRD-Nrg1/H11002 (C, F, and I) mice. Note selective absence of Schwann cells in adductor and gracilis muscles of Isl1Cre/Nrg1flox/H11002 mutant mice and absence of Schwann cells in all muscles of CRD-Nrg1/H11002 mutant mice. A similar phenotype was already observed at E14.5 to E15.5 (data not shown).

Scale bar equals 40 μm.

These findings confirm and extend previous analyses of the pattern of expression of the Types I, II, and III Nrg1 isoforms, which have revealed expression of the Type I isoform in a subset of embryonic DRG neurons (Meyer et al., 1997). Our results provide evidence that the Type I group of Ig domain-containing isoforms of Nrg1 are restricted to proprioceptive sensory neurons. The number of DRG neurons that express Ig domain-containing isoforms, however, appears lower than the total number of TrkC^{+} proprioceptive sensory neurons, raising the possibility that only a subset of proprioceptors express Ig domain isoforms of Nrg1. In this context, the complete loss of muscle spindles in neuronal Nrg1 mutants indicates that all group Ia proprioceptive neurons express Ig domain isoforms, raising the possibility that those proprioceptors that lack Ig domain Nrg1 isoform expression correspond to group I b afferents.

A comparison of the state of muscle spindle differentiation in two different mouse Nrg1 mutants provides a second, genetic, line of evidence that Ig domain iso-
forms of Nrg1 are sufficient, and CRD-containing isoforms are dispensable for the initiation of muscle spindle differentiation. Deletion of all isoforms of Nrg1 from motor and sensory neurons leads to a severe impairment of muscle spindle differentiation, as assessed by the absence of expression of Egr3, Pea3, and Erm and the elaboration of proprioceptive afferent terminals. In contrast, elimination of the CRD-Nrg1 isoforms from sensory and motor neurons (as well as from all other cells) does not affect the early differentiation of muscle spindles. Since both the CRD-Nrg1 and Ig-Nrg1 isoforms are expressed by proprioceptive sensory neurons, comparison of the phenotype of the two Nrg1 mutants suggests two possible roles for Nrg1 isoforms in muscle spindle induction. The Ig-Nrg1 isoforms could be the relevant mediators of proprioceptive afferent fiber inductive activity. Alternatively, Ig-Nrg1 and CRD-Nrg1 isoforms could function in a redundant manner in this inductive process. The lack of an inductive influence of CRD-Nrg1-rich, Ig-Nrg1-poor motor axons on early muscle spindle differentiation provides indirect evidence in favor of the first possibility.

Figure 8. CRD-Nrg1 Mutant Mice Do Not Exhibit a Defect in Early Muscle Spindle Differentiation

Immunocytochemical detection of PV+ proprioceptive afferents at developing muscle spindles (A and E) and in situ hybridization analysis of Egr3 (B and F), Pea3 (C and G), and Erm (D and H) in intrafusal muscle fibers of E16.5 hindlimb muscles of wild-type (A–D) and CRD-Nrg1−/− (E–H) mice. Scale bar equals 20 μm in (A) and (E) and 65 μm in (B)–(D) and (F)–(H).

Figure 9. No Defect in Central Projections of Proprioceptive Afferents in Isl1Cre/Nrg1flox−/− Mutant Mice

Central projections of proprioceptive afferents in E16.5 (A and B) and E18.5 (C and D) lumbar spinal cord of wild-type (A and C) and Isl1Cre/Nrg1flox−/− mutant (B and D) mice were traced by expression of PV. Scale bar equals 115 μm.
Neuregulin-1 and Muscle Spindle Differentiation

Figure 10. Nrg1 Expression by Proprioceptive Afferents Is Critical for the Initiation of Muscle Spindle Differentiation

Summary diagrams of the developmental transition of an unspecified myotube to a fully differentiated muscle spindle (A–D) and accompanying molecular signals involved in the specification of intrafusal muscle fibers (E–H).

(A and E) Before invasion of la afferents into a muscle, intrafusal and extrafusal muscle fibers cannot be distinguished molecularly and both express ErbB receptors.

(B and F) la afferents expressing Nrg1 contact intrafusal muscle fibers, activate ErbB receptor complexes (red arrow in F), and initiate muscle spindle differentiation.

(C and G) After initial contact of la afferents with prospective intrafusal muscle fibers, proprioceptive afferents branch extensively (C), and signaling events downstream of Nrg1/ErbB-receptor complexes result in the induction of the transcription factors Egr3, Pea3, Erm, and factor(s) X (G). A retrograde signal Y from the intrafusal muscle fibers may promote elaboration of annulospiral endings of proprioceptive afferents.

(D and H) During the first postnatal week, muscle spindles are still dependent on continued innervation by proprioceptive afferents (maintenance) and more genes expressed by intrafusal but not extrafusal muscle fibers are expressed (e.g., Er81 and NT-3). NT-3 is thought to act retrogradely to influence the strength of central connections of la afferents with motor neurons (gray arrow).

The idea that the Ig-Nrg1 isoform is the relevant inducer of muscle spindle differentiation is consistent with other studies that have implicated divergent roles for Nrg1 isoforms in cell differentiation. For example, genetic studies in mice have implicated Ig-Nrg1 isoforms in cardiac development, whereas CRD-Nrg1 isoforms are essential for the differentiation and survival of Schwann cells in peripheral nerve (Kramer et al., 1996; Garratt et al., 2000; Wolpowitz et al., 2000). In addition, different Nrg1 isoforms appear to have differential effects on the level of expression of different subunits of nicotinic AChRs and GABA receptors (Yang et al., 1998) as well as NMDA-receptor subunits (Ozaki et al., 1997). Since CRD-Nrg1 and Ig-Nrg1 isoforms appear to be coexpressed by proprioceptive afferents, our experiments, taken together with the genetic analysis of Nrg1 function in Schwann cell differentiation, raise the possibility that different isoforms of Nrg1 expressed by the same neuronal population exert distinct signaling activities on different target cell populations.

How does Nrg1 signaling promote the differentiation of muscle spindles? Importantly, we find that the initiation of muscle spindle differentiation is not impaired in CRD-Nrg1 mutants, mice in which the proliferation and differentiation of peripheral Schwann cells is severely affected (Wolpowitz et al., 2000). This finding suggests a direct interaction between sensory neuron-derived Nrg1 and nascent intrafusal muscle fibers, rather than an indirect action mediated through Schwann cells. All Nrg1 isoforms appear to transduce their biological activities through the activation of heterodimeric transmembrane tyrosine kinase receptors of the ErbB class (Burden and Yarden, 1997), suggesting an involvement of ErbB signaling in the initiation of muscle spindle differentiation. In support of this idea, we detected expression of ErbB3 and ErbB4 in late embryonic intrafusal muscle fibers. In addition, ErbB2 is expressed in adult intrafusal muscle fibers (Andrechek et al., 2002), and the selective elimination of ErbB2 from skeletal muscle fibers results in the absence of mature muscle spindles and in a severe defect in proprioception in adult mice (Andrechek et al., 2002). It remains to be established, however, whether ErbB2 signaling mediates the embryonic role of Nrg1 signaling, although this seems likely.

The role of Nrg1 supplied by proprioceptive afferent fibers in the induction of intrafusal fiber differentiation in muscle spindles offers an informative parallel with the proposed role of Nrg1 supplied by motor axons in the postsynaptic differentiation of extrafusal skeletal muscle fibers at the neuromuscular junction. A series of gain of function studies have shown that both Ig domain and CRD domain isoforms of Nrg1 can activate ErbB signaling in muscle and can activate ETS-containing transcriptional complexes such as GABP, which control expression of nicotinic AChR subunit genes (Sandrock et al., 1997; Schaeffer et al., 1998; Fromm and Burden, 1998; Sapru et al., 1998; Briguet and Ruegg, 2000; Buonanno and Fischbach, 2001). Nevertheless, the role of Nrg1 signaling at developing neuromuscular junctions...
has not been completely resolved: elimination of all neuronal isoforms of Nrg1 in mice does not markedly change the pattern of expression of AChR genes in postsynaptic skeletal muscle (Yang et al., 2001). The loss of muscle spindle differentiation, therefore, provides the clearest example to date of a requirement for neuronal Nrg1 signaling in the differentiation of skeletal muscle fibers.

Impaired Branching of Peripheral Proprioceptive Sensory Terminals in Nrg1 Mutant Mice

Neuronal Nrg1 mutants exhibit an impairment in the elaboration of the peripheral annulospiral branches of group Ia proprioceptive afferent terminals. This axonal branching phenotype is observed in IsI1CreNrg1flox/flox mutants but not in CRD-Nrg1 mutants, and thus is tightly linked to the absence of muscle spindle differentiation. Consistent with this view, no defect in PV− terminal axon morphology is evident at presumed group Ib proprioceptive afferent endings associated with nascent GTOs (Zelea, 1994).

These observations raise the question of how the loss of neurally derived Nrg1 signaling regulates the morphology of proprioceptive afferent terminals. Four possibilities can be considered. First, neurally derived Nrg1 might act in an autocrine manner to stimulate proprioceptive axonal branching directly. Such Nrg1-dependent signaling responses should, classically, be mediated through ErbB receptors, but these receptors are not known to be expressed by DRG neurons (Garratt et al., 2000). Second, peripheral Schwann cells have been shown to depend on Nrg1 for survival (Garratt et al., 2000; Kopp et al., 1997). This observation raised the possibility that Nrg1 may control muscle spindle differentiation through effects on Schwann cell survival. Against this idea, the initiation of muscle spindle differentiation is not impaired in CRD-Nrg1 mutants in which peripheral Schwann cells are severely affected, and conversely in IsI1CreNrg1flox/flox− mutants there are no Schwann cell defects in many of the muscles that lack muscle spindles. Thus, the fate of muscle spindles appears independent of the presence of Schwann cells. Third, there is emerging evidence that certain transmembrane isoforms of Nrg1 can function as receptors in an inverse signaling mode (J. Bao et al., 2001, Soc. Neurosci., abstract), similar to that proposed for Ephrin-Eph kinase signaling (Holland et al., 1996). However, studies on inverse Nrg1 signaling have so far focused on CRD-containing Nrg1 isoforms (J. Bao et al., 2001, Soc. Neurosci., abstract), which are not required for proprioceptive afferent terminal branching. It remains unclear whether Ig-Nrg1 isoforms can also participate in such inverse signaling. A fourth possibility, and one that we favor, is that a retrograde signal provided by nascent muscle spindles induces the branching of proprioceptive afferent terminals. Such retrograde signals may be induced as part of the early intrinsic program of muscle spindle differentiation, in parallel with or downstream of the expression of Egr3, Pea3, and Ern.

One secreted signaling molecule known to be expressed by developing muscle spindles is NT-3 (Copray and Brouwer, 1994; Chen et al., 2002). Reciprocal cell-cell interactions involving Nrg1 and NT-3 have been documented between neuroblasts and nonneuronal cells during early stages of sympathetic neurogenesis (Verdi et al., 1996). However, NT-3 is unlikely to be the relevant retrograde signal directing proprioceptor terminal branching, since its expression in muscle spindles only becomes evident at E18 (Copray and Brouwer, 1994; our unpublished data), well after the defect in proprioceptive afferent branching. The detection of a defect in the branching of group la proprioceptive afferent terminals in the periphery raises the issue of whether a similar defect in branching is evident at the central terminals of these neurons, in the ventral spinal cord. Indeed, loss of the Nrg1-activated ETS factor Er8f1 from muscle spindles and proprioceptive sensory neurons results in a marked defect in the projection of group la afferents into the ventral spinal cord (Arber et al., 2000). In contrast, we have found that the loss of neuronal Nrg1 does not result in any obvious projection or branching defect at the central terminals of proprioceptive afferents in the ventral spinal cord. We have not assayed whether synaptic transmission between proprioceptive afferents and motor neurons is affected in neuronal Nrg1 mutants, although this is likely since there is a marked impairment in monosynaptic sensory-motor transmission associated with the degeneration of muscle spindles in Egr3 mutants (Chen et al., 2002; see also Mendell et al., 2001).

More generally, our findings add to the emerging evidence that Nrg1-like proteins have evolutionarily conserved roles in the induction of target cells, both in neural and nonneural systems. In Drosophila, a neuregulin-like factor, vein, serves an inductive signaling function in muscle and tendon differentiation (Volk, 1999) and regulates the differentiation and survival of glial cells (Hidalgo et al., 2001). Expression of a structurally related EGF-receptor ligand, spitz, is expressed by retinal axons and helps organize postsynaptic cell clusters in the medulla (Huang et al., 1998). Moreover, in C. elegans, the EGF-repeat-containing ligand LIN-3 has a key role in directing the distinct fates of vulval precursor cells through pathways that involve induction of ETS proteins (Katz et al., 1995; Tan et al., 1998). Further studies on the role of Nrg1 isoforms in muscle spindle differentiation may therefore reveal principles of Nrg class signaling that are pertinent to other vertebrate systems and to other organisms.

Experimental Procedures

Mouse Genetics

IsI1CreNrg1flox/flox− mutant mice were generated by setting up timed pregnancies as described by Yang et al. (2001). CRD-Nrg1−/− mutant mice were obtained from L.W. Role, Columbia University, New York (Wolpertowitz et al., 2000). TrkC−/− mice were obtained from L. Parada, University Texas Southwest, Dallas (Liebel et al., 1997), Ngn1−/− mice were provided by D.J. Anderson, CALTECH, Pasadena (Ma et al., 1999), and Egr3−/− mice were provided by W.G. Tourtellotte, Northwestern University, Chicago (Tourtellotte and Milbrandt, 1998). Pea3 mutant mice have previously been described (Livet et al., 2002). Heterozygous animals were interbred to generate mutant embryos of different developmental stages.

In Situ Hybridization and Immunocytohistochemistry

For in situ hybridization analysis, sections were hybridized with digoxigenin-labeled probes (Schaeren-Wiemers and Gerfin-Moser, 1993) directed against mouse Egr3, Pea3 (Livet et al., 2002), Ern (cDNA kindly provided by J.J. Hassell), IsI1, TrkC (Arber et al., 2000), Ig-Nrg1−, and CRD-Nrg1-specific Nrg1 isoforms (Wolpertowitz et al., 2000). For generation of an Egr3-specific probe, a fragment encom-
passing nucleotides (454–1071) of the Egr3 coding sequence (GenBank accession number AF132128) was amplified from genomic DNA by PCR. Antibodies used in this study were: rabbit anti-Egr3, anti-GAP-43, anti-PV, anti-S100, guinea pig anti-Isl1 (Arber et al., 2000), rabbit anti-ErbB3 (Santa Cruz, SC285), anti-ErbB4 (#618; Zhu et al., 1995), and goat anti-PV (SWANT).

Isl1 is expressed transiently by all motor neurons at cell cycle exit, but at later embryonic stages expression in motor neurons innervating limbs is maintained only in motor neurons of the medial subdivision of the lateral motor column (LMCm), those that innervate ventrally located limb muscles (Tsuchida et al., 1994; Arber et al., 1999). In contrast, expression of Isl1 is rapidly downregulated in motor neurons of the lateral LMC that innervate dorsal limb muscles (Tsuchida et al., 1994; Arber et al., 1999; Kania et al., 2000). Expression of Isl1 in all DRG sensory neurons persists from early postmitotic stages, up to at least P10 (Arber et al., 2000). Isl1-directed Cre-recombinase expression in motor and DRG neurons may therefore be expected to act most efficiently in LMCm motor neurons and DRG neurons.

Cryostat sections were processed for immunohistochemistry as described (Arber et al., 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes) (1:1000). Alexa488-labeled α-bungarotoxin (Molecular Probes) was used at 1:2000. Images were collected on an Olympus confocal microscope. Images from in situ hybridization experiments were collected with an RT-SPOT camera, and Corel Photo Paint 10.0 was used for digital processing of images.

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