Transcriptional regulation of myotube fate specification and intrafusal muscle fiber morphogenesis

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Vertebrate muscle spindle stretch receptors are important for limb position sensation (proprioception) and stretch reflexes. The structurally complex stretch receptor arises from a single myotube, which is transformed into multiple intrafusal muscle fibers by sensory axon–dependent signal transduction that alters gene expression in the contacted myotubes. The sensory-derived signal transduction pathways that specify the fate of myotubes are very poorly understood. The zinc finger transcription factor, *early growth response gene 3* (*Egr3*), is selectively expressed in sensory axon–contacted myotubes, and it is required for normal intrafusal muscle fiber differentiation and spindle development. Here, we show that overexpression of *Egr3* in primary myotubes in vitro leads to the expression of a particular repertoire of genes, some of which we demonstrate are also regulated by Egr3 in developing intrafusal muscle fibers within spindles. Thus, our results identify a network of genes that are regulated by Egr3 and are involved in intrafusal muscle fiber differentiation. Moreover, we show that Egr3 mediates myotube fate specification that is induced by sensory innervation because skeletal myotubes that express Egr3 independent of other sensory axon regulation are transformed into muscle fibers with structural and molecular similarities to intrafusal muscle fibers. Hence, *Egr3* is a target gene that is regulated by sensory innervation and that mediates gene expression involved in myotube fate specification and intrafusal muscle fiber morphogenesis.

Introduction

Muscle spindles are stretch receptors that mediate myotactic stretch reflexes as well as limb and axial body position sensation (proprioception) in vertebrates. They consist of specialized, encapsulated muscle fibers (intrafusal muscle fibers) that are innervated by sensory (groups Ia and II) and motor (fusimotor) axons to form mechanoreceptors that relay skeletal muscle stretch sensation to the central nervous system. Late prenatal or early postnatal sensory denervation, but not motor denervation, of developing skeletal muscles leads to spindle degeneration, which indicates that normal spindle development is dependent on sensory, but not fusimotor, innervation (Zelená, 1957; Zelená and Soukup, 1973; Kucera and Walro, 1992). Spindle morphogenesis requires intact sensory innervation, and this has been convincingly demonstrated in mice that lack either neurotrophin-3 (NT-3) or its cognate, tyrosine kinase receptor/TrkC (Ernfors et al., 1994; Klein et al., 1994; Tessarollo et al., 1994). Proprioceptive sensory neuron survival during development depends on NT-3–tyrosine kinase receptor C signaling, and when it is abrogated in mutant mice Ia-afferents do not form properly to induce spindle morphogenesis in the periphery. Thus, Ia-afferent–derived signaling has been recognized as an essential aspect of muscle spindle induction for several decades, but not until recently have some specific signaling mediators been identified. For example, neuregulin-1 (Nrg1) appears to be an essential Ia-afferent–derived mediator of spindle morphogenesis, and although Ia-afferents establish contact with myotubes, muscle spindle morphogenesis and some intrafusal muscle fiber–specific genes are not appropriately induced in mice when sensory neurons are deficient in *Nrg1* (Hippenmeyer et al., 2002). Similarly, muscle spindle morphogenesis is impaired in mice with a muscle-specific deficiency of erythroblastic leukemia viral oncogene homologue 2 (ErbB2), an essential component of the Nrg1 tyrosine kinase receptor signaling complex (Andrechek et al., 2002; Leu et al., 2003). Together, these results indicate that Ia-afferents induce spindle morphogenesis by releasing Nrg1 to engage ErbB2-dependent signal transduction in a subpopulation of myotubes that receive sensory myoneural contacts.

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Abbreviations used in this paper: *Arc, activity-regulated cytoskeletal-associated protein*; DRG, dorsal root ganglion; E, embryonic gestational day; *Egr3, early growth response gene 3*; ErbB2, erythroblastic leukemia viral oncogene homologue 2; ERE, early growth response element; *ERM, Ets-related protein*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *GDNF, glial-derived neurotrophic factor*; *GPR50, g-coupled protein receptor 50*; *Hey1, harry enhance of split 1*; HSA, human skeletal actin; MOI, multiplicity of infection; *NGFR (p75), low affinity nerve growth factor receptor*; Nrg1, neuregulin-1; *NT-3, neurotrophin-3*; *Prph1, peripherin 1*; Pv, parvalbumin; *Rhpn2, rhophilin 2*; Sd-MyHC, slow developmental myosin heavy chain; *SSTr2, somatostatin receptor type 2*.

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Comparatively little is known about the gene regulatory networks that are engaged by ErbB2 signaling in Ia-afferent–contacted myotubes in order to induce their transformation to intrafusal muscle fibers. Presumably, de novo gene expression is required after sensory myoneural contact is established to specify the fate of myotubes that will become morphologically and biochemically distinct intrafusal muscle fibers; to mediate terminal Schwann cell differentiation that will generate the fusiform spindle capsule; and to regulate growth factors that are induced to establish and/or maintain specialized, spindle-related sensory and motor innervation. Accordingly, several transcriptional regulators are induced in myotubes shortly after they are contacted by Ia-afferents, including some Ets-related transcription factors, *ER81* (Arber et al., 2000), Pea3 (Livet et al., 2002), *Ets-related protein* (*ERM*; Hippenmeyer et al., 2002), and the zinc finger transcription factor *early growth response gene 3* (*Egr3*; Tourtellotte and Milbrandt, 1998). They are all regulated by Ia-afferent–derived Nrg1 after myotube contact (Hippenmeyer et al., 2002), indicating that they are candidate transcription factors that are involved in ErbB2-dependent and myotube-intrinsic gene expression related to spindle morphogenesis. However, whether Ets-related transcription factors regulate gene expression that is required for spindle morphogenesis has been difficult to discern. For example, ER81 is expressed in developing spindles, proprioceptive sensory neurons, and motor neurons. In *ER81*-deficient mice, the spindle number is altered in some muscles because ER81 has a role in the specification of some proprioceptive neurons (Kucera et al., 2002). In contrast, muscle spindles show no obvious defects in *Pea3*-deficient mice, despite the fact that it is also coordinately expressed by intrafusal muscle fibers, motor neurons, and sensory neurons (Livet et al., 2002). Finally, ERM expression is not restricted to spindles in the developing muscle, and its role in muscle spindle morphogenesis has not been studied because *ERM*-deficient mice die before muscle spindle induction (Hippenmeyer et al., 2002).

Egr3 is a particularly interesting candidate effector molecule of Nrg1–ErbB2 signaling in myotubes. Egr3 is induced in Ia-afferent–contacted myotubes at a developmental time point that coincides with Ia-afferent innervation, and it is not expressed by sensory or motor neurons that innervate them. Moreover, Ia-afferent–contacted myotubes in *Egr3*-deficient mice fail to differentiate into intrafusal muscle fibers that express an intrafusal muscle fiber–specific, slow developmental myosin heavy chain (Sd-MyHC; Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001), suggesting that their differentiation may be impaired. As one of the earliest recognized transcriptional regulators that are induced selectively in myotubes by Ia-afferent innervation, Egr3 may mediate some aspects of Nrg1–ErbB2 signaling that is relevant to myotube fate specification and intrafusal fiber differentiation. However, neither the target genes regulated by Egr3 in Ia-afferent–contacted myotubes nor its potential role in myotube fate specification have ever been directly examined. In this study, we identified many target genes that are regulated by Egr3 in primary myotubes in vitro and demonstrated that some of these genes are regulated by Egr3 in developing spindles in vivo. To examine whether Egr3 has any instructive role in specifying the fate of myotubes to become intrafusal muscle fibers, transgenic mice were generated to express Egr3 in all myotubes independent of Ia-afferent–Nrg1 signaling. These mice were not viable because their skeletal muscle fibers were entirely transformed to contain muscle fibers that were structurally and biochemically similar to intrafusal muscle fibers. Considered together, these results demonstrate that Ia-afferent–Nrg1 signaling in myotubes serves, at least in part, to regulate *Egr3* as an important transcriptional regulator of myotube fate specification and intrafusal muscle fiber morphogenesis.

Results

Egr3-mediated gene regulation in primary murine myotubes

Egr3 expression is regulated in myotubes by sensory innervation and Nrg1 signaling in vivo (Tourtellotte et al., 2001; Hippenmeyer et al., 2002; Jacobson et al., 2004). Ia-afferents contact myotubes in *Egr3*-deficient mice; in the absence of *Egr3*-mediated gene regulation, they do not properly differentiate into spindles, which leaves the mice with profound sensory ataxia and proprioceptive deficits (Tourtellotte and Milbrandt, 1998). To identify target genes that are potentially regulated by Egr3 in Ia-afferent–contacted myotubes, primary myoblasts were isolated from newborn, wild-type mice and were differentiated into multinucleated myotubes. One population of myotubes was infected with an adenovirus that coexpressed enhanced GFP (EGFP) and full-length, transcriptionally active wild-type Egr3 ($Egr3_{WT}$), whereas another population of myotubes was infected with an adenovirus that coexpressed EGFP and transcriptionally inactive truncated Egr3 ($Egr3_{Tr}$; Fig. 1 A). Primary myotubes that were infected with 100% efficiency expressed high levels of transgenic protein and showed no evidence of toxicity (Fig. 1 B). Endogenous Egr3 was not detected in cultured myotubes, similar to myotubes that neither receive Ia-afferent innervation nor become intrafusal muscle fibers in vivo (Fig. 1 B, bottom right; Tourtellotte and Milbrandt, 1998). To identify genes regulated by Egr3 in this primary myotube–cellular context, gene expression in the adenovirus-infected myotubes was compared using Affymetrix microarray analysis. The experiment was repeated twice, and a four-way analysis of the gene expression datasets identified 83 unique genes that were up-regulated and five unique genes that were down-regulated by $Egr3_{WT}$ in primary myotubes. Egr3 appears to be a transcriptional activator in the cellular contexts where it has been studied, which is consistent with the observation that many more genes were up-regulated by $Egr3_{WT}$ (Table I, 83 genes, 2–244-fold) than were down-regulated (Table II, five genes, two- to threefold). Although most of the 83 genes identified have not been evaluated further, up-regulation was confirmed in 15/15 genes examined by using a real-time PCR analysis on cDNA samples that were prepared from independently infected primary myotubes (Fig. 1 D, results from 12 genes shown; Table I).

Egr3-mediated gene regulation in developing muscle spindles

To examine whether Egr3 target genes identified in vitro may also be regulated by Egr3 in a similar cellular context in devel-

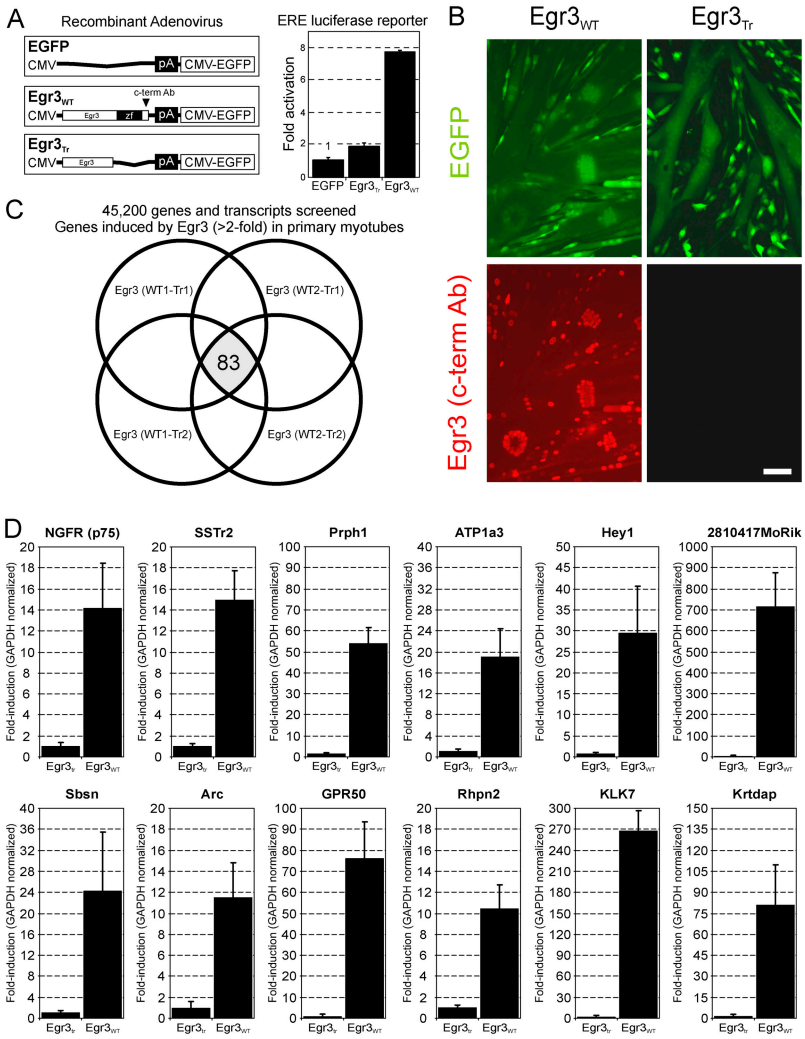

Figure 1. **Identification of Egr3 target genes in primary murine myotubes.** (A) Adenoviruses were generated to express either EGFP alone (EGFP), full-length, transcriptionally active Egr3 and EGFP ($Egr3_{WT}$), or transcriptionally inactive Egr3 and EGFP ($Egr3_{Tr}$). The transcriptional activity of the recombinant proteins that were produced by the viruses was assessed by the early growth response element (ERE) luciferase reporter assay. Compared with EGFP and $Egr3_{Tr}$-infected myoblasts, $Egr3_{WT}$ infection showed marked transcriptional activation of the ERE–luciferase reporter plasmid (mean and standard deviation shown from two independent transfection-infection experiments, each performed in triplicate). (B) Myotubes were differentiated for 10 d and infected with either the $Egr3_{WT}$ or $Egr3_{Tr}$ adenovirus. EGFP fluorescence confirmed 100% infection efficiency, and immunohistochemistry using a COOH-terminal Egr3 antibody demonstrated high levels of nuclear localized Egr3 in $Egr3_{WT}$-infected myotubes and no staining in the $Egr3_{Tr}$-infected myotubes. Results from the $Egr3_{Tr}$-infected cells further demonstrated the lack of endogenous Egr3 expression in wild-type myotubes. Bar, 40 μm. (C) Total RNA was extracted from the myotubes 18 h after infection, and Affymetrix microarray analysis was performed. The infection experiments were performed twice, and the gene expression datasets were compared in a four-way analysis. 83 genes were identified as up-regulated in all of the single, four-way comparisons from 45,200 genes and transcripts that were analyzed. (D) All of the genes tested (15/15) were confirmed to represent up-regulated genes by real-time PCR (mean and standard deviation from four independent infection experiments and real-time PCR assays, each performed in triplicate, $P < 0.05$ for all genes).

oping muscle spindles in vivo, we used in situ hybridization to characterize gene expression patterns in embryonic wild-type mouse muscles. Egr3 and Pea3 are transcription factors regulated by Ia-afferent–Nrg1 signaling in myotubes, and Ia-afferents contact myotubes in both *Egr3*-deficient and *Pea3*-deficient mice (Tourtellotte and Milbrandt, 1998; Livet et al., 2002). Pea3 is not necessary for spindle morphogenesis, but it is a useful marker for localizing Ia-afferent–contacted myotubes in the developing skeletal muscle (Hippenmeyer et al., 2002). We found that Pea3 was not regulated by Egr3 because Pea3 expression was identified in discretely labeled myotubes in *Egr3*-deficient mice (Hippenmeyer et al., 2002). However, Pea3-expressing myotubes were reduced by ~66% in Egr3-deficient muscles at the embryonic gestational day (E) 16.5 (Fig. 2 A) and were reduced by ~86% at E18.5 (Fig. 2 B). Because Pea3 is a marker of Ia-afferent–myotube contact, the decreased number of Pea3-expressing myotubes in *Egr3*-deficient mice is consistent with previous observations that demonstrate initial Ia-afferent contact of myotubes, progressive loss of afferents during prenatal development, and complete loss of afferents in adult *Egr3*-deficient mice (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Because some Ia-afferent–contacted, Pea3-expressing myotubes were present in prenatal *Egr3*-deficient muscles, it was possible to examine potential Egr3 target gene expression within them. Accordingly, Egr3-dependent gene expression in skeletal muscle should be restricted to Ia-afferent–contacted myotubes where Egr3 expression is restricted, and it should be abrogated in Pea3-expressing (Ia-afferent contacted), *Egr3*-deficient myotubes. We examined six target genes that were identified by Affymetrix microarray analysis (and were confirmed to be up-regulated in myotubes by real-time PCR analysis) using in situ hybridization on the muscle. Although three genes (*rhophilin 2* [*Rhpn2*]; *g-coupled protein receptor 50* [*GPR50*]; and *activity-regulated cytoskeletal-associated protein* [*Arc*]) showed no specific localization to muscle spindles (unpublished data), the three other genes, including two growth factor receptors (*low affinity nerve growth factor receptor* [*NGFR (p75)*]; and *somatostatin receptor type 2* [*SSTr2*]) and the type III intermediate filament *peripherin 1* (*Prph1*), appeared to be expressed selectively by spindles. In E18.5 wild-type skeletal muscle, NGFR (p75) (Fig. 3 A), SSTr2 (Fig. 3 B), and Prph1 (Fig. 3 C) expression was restricted to muscle spindles similar to the expression pattern of Egr3. In skeletal muscles from E18.5 *Egr3*-deficient mice, NGFR (p75), SSTr2, and Prph1 were not expressed in Pea3-expressing myotubes (Fig. 3, A–C, bottom). Hence, NGFR (p75), SSTr2, and Prph1 represent new

Table I. **Up-regulated genes ($Egr3_{WT}$ relative to $Egr3_{Tr}$)**

Affymetrix fold change ($n = 4$)	Up-regulated in myotubes >fivefold ($n = 4$)	Up-regulated in HSA–*Egr3* mice >fivefold ($n = 4$)	Expression spindles	Title	Gene symbol[a]	NCBI accession no.[b]
244.04	+	+	−	*rhophilin, Rho GTPase-binding protein 2*	*Rphn2*	BF228009
56.16	+	+	−	*G-coupled protein receptor 50*	*GPR50*	BQ032613
44.94	+	+		RIKEN *cDNA 2810417M05 gene*	*2810417M05Rik*	AK014196.1
38.87				*plasmalemma vesicle-associated protein*	*Plvap*	NM_032398.1
29.07	+			*synaptosomal-associated protein 25*	*Snap25*	BC018249.1
27.94	+	+	+	*peripherin 1*	*Prph1*	NM_013639.1
27.33	+			*ubiquitin protein ligase E3A*	*Ube3a*	BB313387
17.63	+			*keratinocyte differentiation associated protein*	*Krtdap*	AV007306
14.37	+			*suprabasin*	*Sbsn*	AI507307
11.91	+	+		*hairy/enhancer-of-split related with YRPW motif 1*	*Hey1*	NM_010423.1
10.62				*cysteine dioxygenase 1, cytosolic*	*Cdo1*	NM_033037.1
10.26				RIKEN *cDNA 1110065A22 gene*	*1110065A22Rik*	BB768838
7.60	+	+	−	*activity-regulated cytoskeletal-associated protein*	*Arc*	NM_018790.1
7.54				*F-box only 2 protein*	*Fbxo2*	BB311718
7.48				*GABA-A receptor, subunit α4*	*GABRA4*	BB430205
7.46	+			*chloride channel 2*	*Clcn2*	NM_009900.1
6.77				*leucine-rich repeat neuronal 6A*	*Lrrn6a*	BB078751
6.63				*elastin microfibril interfacer 3*	*Elimin3*	BG076147
6.61				*ribosomal protein S6 kinase polypeptide 1*	*Rps6ka1*	NM_009097.1
6.48				*dermokine; epidermal secreted protein*	*Dmkn*	BI452905
6.33				*keratin complex 1, acidic, gene 18*	*Krt1-18*	NM_010664.1
6.23	+	+	+	*somatostatin receptor 2*	*Sstr2*	NM_009217.1
5.97	+	−		*neuronal d4 domain family member*	*Neud4*	AW553317
5.94	+	+		*ATPase, Na^+/K^+ transporting, α3 polypeptide*	*Atp1α3*	BC027114.1
5.81				*IKKb, epsilon*	*Nfkbie*	BB820441
5.73				*brain-specific protease-4 (BSSP-4)*	*Prss22*	NM_133731.1
5.62				*hypothetical protein LOC327991*	*LOC327991*	BB346082
5.59	+	+	+	*nerve growth factor receptor (p75)*	*Ngfr*	BB151515
5.23				*ESTs*	—	BB251859
5.19				RIKEN *cDNA A030009H04 gene*	*A030009H04Rik*	NM_020591.1
4.99				*inhibin α*	*Inhα*	NM_010564.1
4.82				RIKEN *cDNA 3110007G05 gene*	*3110007G05Rik*	BE992549
4.78				*procollagen, type II, α1*	*Col2α1*	NM_031163.1
4.49				*serologically defined colon cancer antigen 33*	*Sdccag33*	AV291373
4.44				*ubiquitin COOH-terminal hydrolase L1*	*Uchl1*	NM_011670.1
4.41				RIKEN *cDNA 8430427H17 gene*	*8430427H17Rik*	BM120675
4.40				*Mus musculus, clone IMAGE:5368459, mRNA*	—	BF468072
4.27				*neural apoptosis regulated convertase 1*	*Narc1*	AV010795
4.25				*leptin receptor*	*Lepr*	BM124366
4.24				RIKEN *cDNA 2210010L05 gene*	*2210010L05Rik*	BC016598.1
4.23				*hairy/enhancer-of-split related with YRPW motif 2*	*Hey2*	NM_013904.1
4.13				RIKEN *hypothetical protein, 6330405H19*	*6330405H19Rik*	BB225226
4.04				*carbonic anhydrase 2*	*Car2*	NM_009801.2
3.94				*GRP1-associated scaffold protein*	*Grasp*	NM_019518.1
3.77				*septin 4*	*Sept4*	AW208509
3.75				RIKEN *hypothetical protein, C130068N17*	*C130068N17Rik*	AW548377
3.55				*neuron-specific enolase 1*	*Nse1*	BC002154.1
3.53				*thiosulfate sulfurtransferase, mitochondrial*	*Tst*	BC005644.1
3.51				*chemokine (C-X3-C motif) ligand 1*	*Cx3cl1*	AF071549.1
3.49				*forkhead box A3*	*Foxa3*	AK019022.1
3.44				*periaxin*	*Prx*	BG072867
3.41				*preproenkephalin 1*	*Penk1*	M13227.1
3.41				*chloride channel K1*	*Clcnk1*	C76734
3.30				*phospholipase Cδ*	*Plcδ*	NM_019676.1
3.21				RIKEN *cDNA clone, A030009H04*	*A030009H04Rik*	AK002962.1
3.19				RIKEN *cDNA clone, 1810017F10*	*1810017F10Rik*	BC019563.1
3.12				*Ets transcription factor Spi-B*	*Spi-B*	BM244106
3.09				RIKEN *cDNA clone, B230331L10*	*B230331L10Rik*	BB357157
3.07				*ESTs*	—	BE948505
3.06				*ephrin A3*	*Efna3*	AA388313
2.99				RIKEN *cDNA clone, E030041H03*	*E030041H03Rik*	BB535044
2.97				*caspase recruitment domain family, member 10*	*Card10*	NM_130859.1
2.96				*elongation of very long chain fatty acids*	*Elovl1*	BB145065
2.96				*amnionless*	*Amn*	NM_033603.1
2.94				*phenylethanolamine-N-methyltransferase*	*Pnmt*	NM_008890.1
2.87				RIKEN *cDNA, 2210010L05*	*2210010L05Rik*	BF225441
2.84				*melanoma cell adhesion molecule*	*Mcam*	NM_023061.1

Table I. **Up-regulated genes ($Egr3_{WT}$ relative to $Egr3_{Tr}$) (continued)**

Affymetrix fold change ($n = 4$)	Up-regulated in myotubes >fivefold ($n = 4$)	Up-regulated in HSA–*Egr3* mice >fivefold ($n = 4$)	Expression spindles	Title	Gene symbol[a]	NCBI accession no.[b]
2.82				*brain-specific angiogenesis inh. 1-associated protein 2*	*Baiap2*	AF390178.1
2.82				*myosin light chain, phosphorylatable, cardiac ventricles*	*Mylpc*	NM_010861.1
2.82				*prostaglandin I receptor (IP)*	*Ptgir*	AV373771
2.80				*transforming growth factor, β1*	*Tgfb1*	NM_011577.1
2.73				*ESTs similar to pentraxin I (NP-I)*	—	AI152800
2.73				*ESTs*	—	AW046863
2.69				*superoxide dismutase 3, extracellular*	*Sod3*	U38261.1
2.69				*fatty acid desaturase 2*	*Fads2*	NM_019699.1
2.65				*syndecan 1*	*Sdc1*	BI788645
2.63				*semaphorin 3B*	*Sema3b*	NM_009153.1
2.62				RIKEN *cDNA, C030047F10*	*C030047F10Rik*	BE996518
2.59				*cullin 4B*	*Cul4b*	AW536452
2.56				RIKEN *cDNA, 9130011B11*	*9130011B11Rik*	BG068678
2.50				RIKEN *cDNA, 2900002H16*	*2900002H16Rik*	NM_021430.1
2.36				*fatty acid desaturase 2*	*Fads2*	BB430611
2.27				*amyloid β (A4) precursor-like protein 1*	*Aplp1*	AI848048

83 genes were up-regulated between 2- and 244-fold by Egr3 in myotubes in all of the four comparative analyses performed from two independent microarray experiments. Some of these genes have been verified as up-regulated by real-time PCR in Egr3-expressing myotubes, in HSA–Egr3 transgenic muscle tissue, and by in situ hyrbridization as indicated. The gene symbol names for all gene entries coincide with the symbols used by the National Center for Biotechnology Information (NCBI) databases.
[a]NCBI unified gene nomenclature according to Entrez Gene.
[b]NCBI GenBank accession number of sequence used to generate gene feature on Affymetrix chip.

intrafusal muscle fiber–restricted genes that are regulated by Egr3. These results indicate that the target genes identified in vitro may at least partially represent the Egr3-mediated gene regulatory network involved in spindle morphogenesis.

A role for Egr3 in myotube fate specification and intrafusal muscle fiber morphogenesis

During skeletal muscle development, Ia-afferents contact a relatively small number of myotubes to engage the Nrg1–ErbB2 signaling that specifies whether they will become intrafusal muscle fibers. Egr3 appears to have a role in regulating at least some target genes that are involved in spindle morphogenesis, but it is not known what role it may have in myotube fate specification that is mediated by Ia-afferent–Nrg1 signaling. To examine whether Egr3 has an instructive role in myotube fate specification, transgenic mice were generated using the human skeletal actin (HSA) promoter to enforce Egr3 expression in all skeletal myotubes independent of Ia-afferent–Nrg1 regulation (Fig. 4 A). It was not possible to generate transgenic founders that could be used to establish lines of transgenic mice because they were not viable. To facilitate the analysis of the transgenic founders, real-time PCR was used to examine the level of Egr3 expression from forelimb-derived RNA in every embryo that was generated. Animals were analyzed based upon their genotype and level of Egr3 expression by grouping them into those that did not carry the transgene (Tg−; wild type), those that carried the transgene but did not express transgenic Egr3 above endogenous levels (Tg+/Φ), those that expressed low levels of transgenic Egr3 (Tg+/L; 3–10-fold increased expression relative to Tg− mice), and those that expressed high levels of transgenic Egr3 (Tg+/H; >10-fold increased expression relative to Tg− mice; Fig. 4 B). Late gestation embryos that expressed the transgene (Tg+/L and Tg+/H) were consistently smaller than their transgene negative (Tg−) or nontransgene-expressing (Tg+/Φ) litter mates (Fig. 4 C). E18.5 transgene-expressing mice had beating hearts, flexion contractures of their extremities, no spontaneous or evoked motor activity, and were not viable at birth (Fig. 4 D). The level of Egr3 expression in particular founder mice correlated with the relative number of muscle fibers that expressed Egr3. Egr3 expression in E18.5 wild-type skeletal muscle was restricted to developing spindles, which were relatively few in number (Fig. 4 E, arrows), whereas Egr3 was expressed by most of the muscle fibers in transgene-expressing mice (Fig. 4 F).

Transgene-expressing mice had poorly formed skeletal muscles that were generally more poorly formed in Tg+/H muscles than in Tg+/L muscles. As expected, muscles from mice that did not express the transgene (Tg+/Φ) appeared normal. In E18.5 wild-type hindlimbs, discrete muscles were readily identified (Fig. 5 A), whereas distinct muscles were largely absent in transgene-expressing hindlimbs (Fig. 5 B). Wild-type muscle fibers contained skeletal myofilaments and subsarcolemmal nuclei that are typical of immature skeletal myofibers (Fig. 5, C and E). Occasional spindles were identified that consisted of intrafusal muscle fibers with scant myo-

Table II. **Down-regulated genes ($Egr3_{WT}$ relative to $Egr3_{Tr}$)**

Affymetrix fold change ($n = 4$)	Title	Gene symbol[a]	NCBI accession no.[b]
−2.94	*Mus musculus n-BAK1 (Bak1)*	*Bak1*	AF402617.1
−2.75	*myogenic factor 6*	*Myf6*	NM_008657.1
−2.71	*acetylcholinesterase*	*Ache*	NM_009599.1
−2.16	*LIM and cysteine-rich domains 1*	*Lmcd1*	BC019124.1
−2.15	RIKEN *cDNA 1110019L22 gene*	*1110019L22Rik*	AK003826.1

Five genes were identified as down-regulated between 2- and 2.9-fold. None of these genes has been investigated further as indicated. The gene symbol names for all gene entries coincide with the symbols used by the National Center for Biotechnology Information (NCBI) databases.
[a]NCBI unified gene nomenclature according to Entrez Gene.
[b]NCBI GenBank accession number of sequence used to generate gene feature on Affymetrix chip.

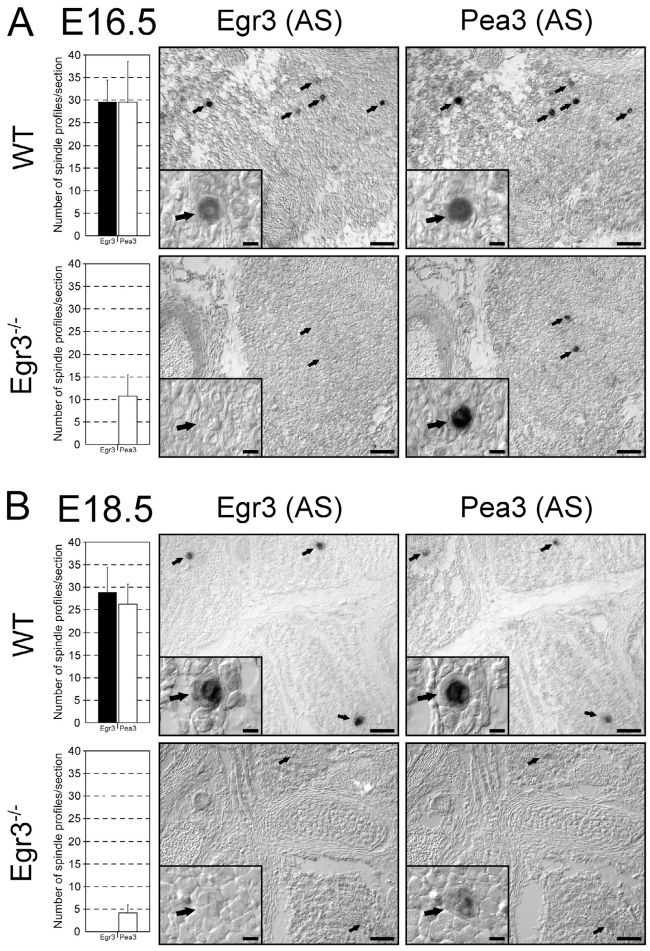

Figure 2. **Pea3 expression in wild-type and *Egr3*-deficient muscle is a marker of Ia-afferent–contacted myotubes in vivo.** (A) At E16.5, shortly after Ia-afferent contact is made with myotubes, Pea3 is expressed in both wild-type and *Egr3*-deficient myotubes. In *Egr3*-deficient muscles, the number of myotubes that express Pea3 is reduced by 66% in E16.5 embryos, and (B) in E18.5 embryos, they are reduced by 86%. Ia-afferents are known to initially contact myotubes in *Egr3*-deficient mice and then withdraw during development, which is consistent with the progressive decrease in Pea3-expressing myotubes that are identified in *Egr3*-deficient muscles. Thus, Pea3 is not regulated by Egr3 in myotubes, and it is a marker for Ia-afferent contact. These results make it possible to use Pea3 to localize Ia-afferent–contacted myotubes and study potential Egr3 target gene expression within them ($n = 3$ animals for each genotype; mean and standard deviation shown). Insets are high power magnifications of labeled spindles (arrows). Bars: 50 μm; (inset) 10 μm.

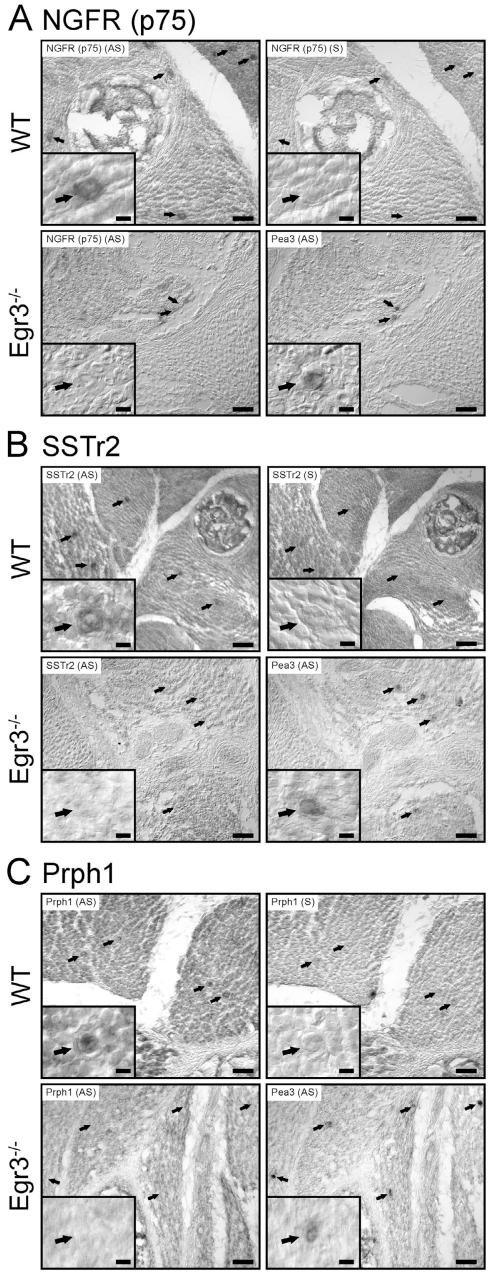

Figure 3. **Egr3 regulates specific target genes in developing spindles.** Affymetrix microarray analysis demonstrated many potential target genes that are regulated by Egr3 in myotubes. Egr3 was necessary for the expression of (A) NGFR (p75), (B) SSTr2, and (C) Prph1 in muscle spindles. The muscle expression of all of these genes was restricted to the developing spindles (arrows), similar to the expression pattern of Egr3, whereas their expression was abrogated in Pea3-expressing (Ia-afferent contacted), *Egr3*-deficient myotubes. Bars: 50 μm; (inset) 10 μm.

fibrillary architecture and internal nuclei. Sections through the equatorial plane of the spindles showed prominent annulospiral axons (Ia-afferents) circumscribing the intrafusal muscle fibers within rudimentary spindle capsules (Fig. 5 E, arrowhead). In contrast, all muscle fibers from transgene-expressing mice contained scant myofilaments with centrally located nuclei. Although most were single multinucleated fibers that had myofibrillary structure and nuclear features indistinguishable from intrafusal muscle fibers, many were aggregated into clusters that were similar to intrafusal muscle fibers within spindles (Fig. 5, D and F [arrow]). Most of the intrafusal-like muscle fibers were not encapsulated or innervated as determined from an analysis of serially sectioned, resin-embedded hindlimbs. Occasional normal-appearing spindles were identified in muscles from transgene-expressing mice that had the typical configuration of three to four intrafusal muscle fibers, which were circumscribed by annulospiral sensory axons and rudimentary capsules (Fig. 5 F, arrowhead). Thus, Egr3 expression in skeletal myotubes independent of other Ia-afferent signaling has the remarkable capacity to transform them into muscle fibers that are structurally similar to intrafusal muscle fibers, presumably orchestrated by the network of genes it regulates.

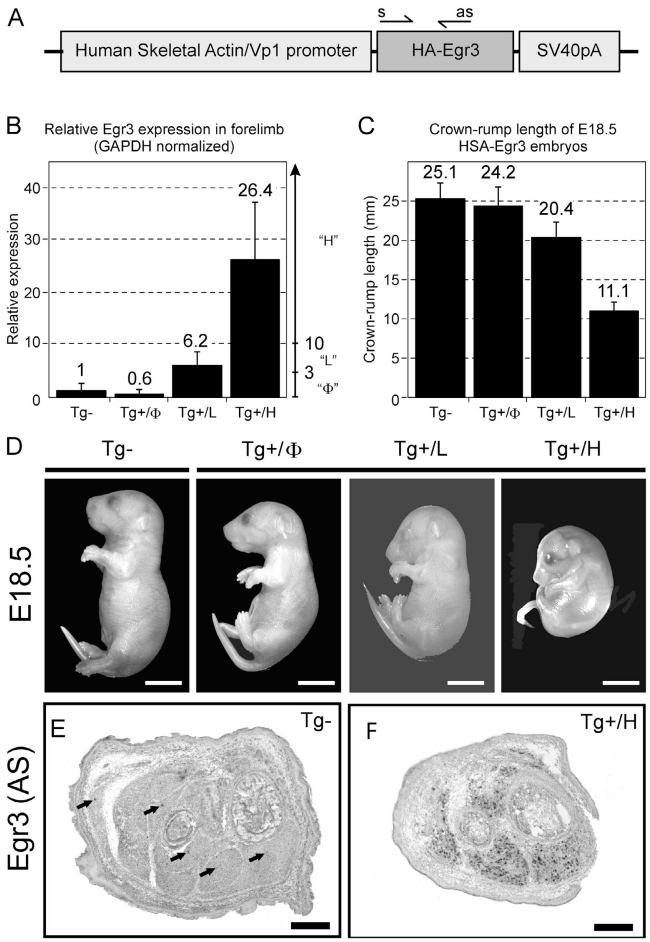

Figure 4. **Enforced Egr3 expression in all skeletal myotubes leads to muscle defects and perinatal mortality.** (A) The human skeletal actin (HSA) promoter was used to express Egr3 in all skeletal myotubes independent of normal Ia-afferent–mediated regulation. PCR was used to genotype the mice using the primers indicated (s and as). (B) Perinatal founder mice were not viable, making it necessary to characterize all of the individual embryos generated from the pronuclear injections. Using real-time PCR to examine the expression of Egr3 in muscle, a total of 58 embryos were studied by separating them into four groups: (1) those that did not carry the transgene (Tg−); (2) those that carried the transgene but did not express it (Tg+/Φ); (3) those that carried the transgene and expressed it at low levels (Tg+/L; 3–10-fold increase in Egr3 expression relative to Tg− muscles); and (4) those that carried the transgene and expressed it at high levels (Tg+/H; >10-fold increase in Egr3 expression relative to Tg− muscles; n = 8–20 embryos per group; mean and standard deviation shown). (C) The transgene-expressing mice were consistently smaller than either their Tg− or Tg+/Φ litter mates (n = 8–20 embryos per group; mean and standard deviation shown). (D) The late gestational (E18.5) Tg+/L and Tg+/H embryos had beating hearts, flexion contractions, and exhibited no spontaneous or evoked motor activity. Bars, 5 mm. (E) In situ hybridization demonstrated normal Egr3 expression in developing spindles in both Tg− and Tg+/Φ muscles (arrows). (F) In contrast, Tg+ mice expressed the transgene in most muscle fibers, many of which were aggregated into fibers with numerous internal nuclei. Bars, 0.5 mm.

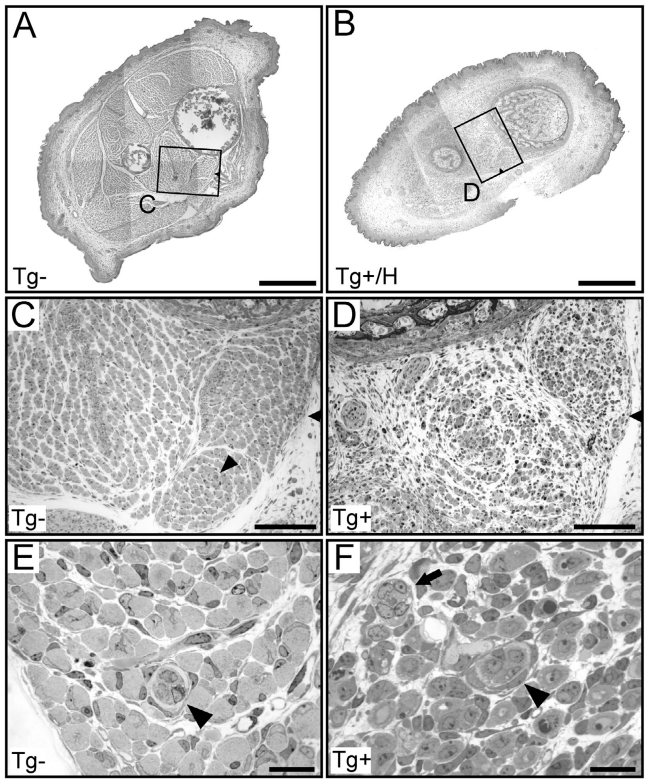

Figure 5. **Skeletal myotubes are transformed into muscle fibers that are structurally similar to intrafusal muscle fibers in HSA–*Egr3* transgenic mice.** E18.5 (A, C, and E) wild-type and (B, D, and F) Tg+/H transgenic hindlimbs. (A) In wild-type embryos, skeletal muscles were discretely formed (lower hindlimb shown), whereas (B) in HSA–*Egr3* transgene-expressing embryos, the individual muscles were poorly distinguished. Boxed areas in A and B are shown in C and D at higher magnifications. (C and E) Discrete wild-type muscles contained normal muscle fibers that were distended by skeletal myofilaments and contained subsarcolemmal nuclei. (C) Occasional normal spindles were noted (arrowhead). (E) They contained three to four intrafusal muscle fibers with scant myofibrillary structure and internal nuclei, which were surrounded by axons (Ia-afferents) and rudimentary capsules (arrowhead). (D) Muscle fibers from transgene-expressing mice had scant myofibrillary structure and internal nuclei similar to those of intrafusal muscle fibers. (F) Many of the fibers were aggregated into spindle-like structures that lacked capsules or obvious innervation (arrow). Scattered, normal-appearing spindles were noted to have capsules and innervation (arrowhead). Bars: (A and B) 500 μm; (C and D) 20 μm; (E and F) 100 μm.

Intrafusal-like muscle fibers in HSA–*Egr3* transgenic muscle are not innervated

Intrafusal-like muscle fibers in Egr3 transgene-expressing muscles do not have fusiform capsules, which may derive from terminal Schwann cells of innervating axons. To examine whether the transformed muscle fibers were innervated by either fusimotor or proprioceptive axons, parvalbumin (Pv) immunohistochemistry was used as a selective marker for proprioceptive neurons and Ia-afferents (Tourtellotte and Milbrandt, 1998), and ATP1α3 immunohistochemistry was used as a marker for both Ia-afferent and fusimotor axons in skeletal muscles (Dobretsov et al., 2003). There was no quantitative difference in the number of proprioceptive neurons in the Tg− or Tg+/H fifth lumbar dorsal root ganglion (DRG; Fig. 6 A), and there was no qualitative difference in the number of Pv+ neurons in any first through fourth cervical or first through fifth lumbar DRG that were examined, suggesting that the number of innervated and structurally normal spindles (Fig. 5 F, arrowhead) was normal in Tg+/H mice. However, whereas robust Pv+ Ia-afferent axons terminated on spindles in Tg− mice (and occasional normal-appearing spindles in Tg+/H mice), the intrafusal-like muscle fibers from Tg+/H mice showed no evidence of Ia-afferent innervation (Fig. 6 B). Similarly, the

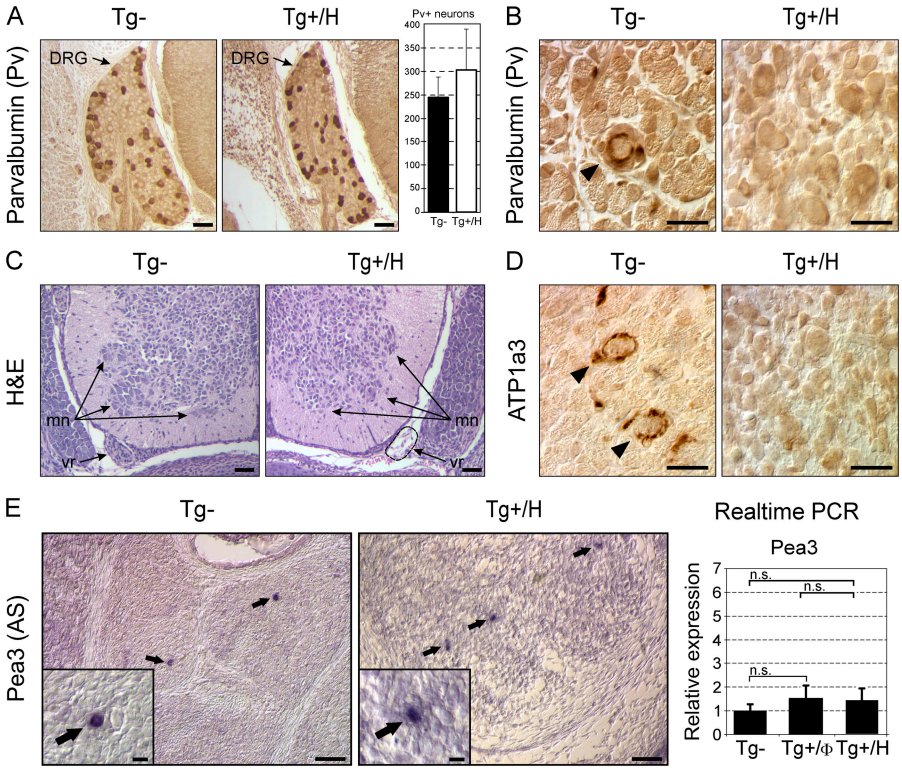

Figure 6. **Intrafusal-like muscle fibers are not innervated in HSA–*Egr3* transgene-expressing mice.** (A) A subset of proprioceptive neurons in the dorsal root ganglia (DRG) give rise to Ia-afferents that express parvalbumin (Pv). There was no difference in the number of Pv+ neurons in DRG between Tg− and Tg+/H mice (fifth lumbar DRG shown), which is consistent with normal innervation of some spindles in Tg+/H muscles (n = 4 for each genotype; mean and standard deviation shown). (B) However, although Pv+ Ia-afferents were robustly labeled in normal spindles in Tg− (arrowhead) and Tg+/H mice (not depicted), there was no evidence of Pv+ axon innervation in any of the transformed myotubes in Tg+/H muscles. (C) Muscles in Tg+/H mice were not capable of sustaining any motor innervation. Motor neurons (mn) present in Tg− spinal cords (lumbar cord shown) were entirely absent in Tg+/H mice, leaving only spinal interneurons in their place. Complete motor neuron loss was corroborated by the absence of ventral roots (vr), which carry motor axons into the periphery. (D) Spindles in Tg− muscles received both Ia-afferent and fusimotor innervation, which was robustly labeled by ATP1α3 immunohistochemistry, whereas no ATP1α3-labeled axons innervated the transformed myotubes in Tg+/H muscles. Arrowheads indicate the spindles innervated by ATPα3-containing axons. (E) Consistent with the observation that Pv+ neurons are not altered in Tg+/H mice, Ia-afferent–contacted, Pea3-expressing myotubes were observed in Tg+/H muscles, and Pea3 expression was not significantly different from wild-type (Tg−) or nontransgene-expressing (Tg+/Φ) muscles (n = 4 for each condition; mean and standard deviation shown). Inset shows a magnified image of the Pea3-expressing spindle (arrow). Bars: (A, C, and E) 50 μm; (B and D) 20 μm; (inset) 10 μm.

transformed muscle fibers were not able to sustain motor innervation as indicated by the near complete loss of motor neurons in the spinal cord and the absent ventral roots in Tg+/H mice (Fig. 6 C). In the periphery, ATP1α3 identified fusimotor and Ia-afferent axons that terminated selectively on normal spindles, whereas there was no evidence of such innervation in the transformed muscle fibers in Tg+/H muscles (Fig. 6 D).

Pea3 is expressed in myotubes that have been contacted by Ia-afferents, but in Tg+/H muscle there was no change in the number of Pea3-expressing myotubes or the expression level of Pea3 in transgene-expressing muscle (Fig. 6 E). These results are consistent with our findings that Pea3 is not regulated by Egr3 (Fig. 2), that some morphologically intact spindles are innervated in transgene-expressing muscles (Fig. 5 F, arrowhead), and that the transformed muscle fibers are not innervated by Ia-afferents that would otherwise be expected to induce high levels of Pea3 expression. Thus, myotubes that express Egr3 in the absence of other Ia-afferent–derived signaling are morphologically similar to intrafusal muscle fibers, but they are devoid of intact fusiform capsules and are incapable of attracting and/or sustaining their innervation.

Muscle fibers from HSA-*Egr3* transgenic mice express genes similar to those of intrafusal muscle fibers

Enforced expression of Egr3 in skeletal myotubes is sufficient to transform them into muscle fibers that structurally resemble intrafusal muscle fibers. To examine whether they also express genes that are characteristic of intrafusal muscle fibers, several well-established intrafusal muscle fiber markers and some newly identified markers were examined in HSA–*Egr3* transgenic muscles. In wild-type muscle, Sd-MyHC is expressed by the Ia-afferent contact of myotubes as one of the earliest markers of intrafusal muscle fiber formation. Sd-MyHC is not expressed in Ia-afferent–contacted, Egr3-deficient myotubes, suggesting that they are impaired in their capacity to differentiate into intrafusal muscle fibers (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Sd-MyHC was localized to intrafusal muscle fibers in developing spindles in wild-type (Tg−) muscle as previously reported, whereas it was expressed by 15-fold more fibers in Tg+/H muscles, indicating that some myotubes had acquired at least one phenotype of intrafusal muscle fibers (Fig. 7 A). The neurotrophins NT-3 and glial-derived neurotrophic factor (GDNF) are preferentially expressed by intrafusal muscle fibers in E18.5 muscle and are required for their sensory and fusimotor innervation, respectively (Ernfors et al., 1994; Whitehead et al., 2005). Relative to Tg− muscles, neither GDNF nor NT-3 was up-regulated in Tg+/Φ muscles, but both were up-regulated 4- and 5.2-fold, respectively, in Tg+/H muscles (Fig. 7 B). The newly identified Egr3 target genes *NGFR (p75)*, *SSTr2*, and *Prph1* were not up-regulated in Tg+/Φ muscles relative to Tg− muscles, whereas they were all up-regulated in Tg+/H muscle 6.4-, 6.7-, and 4-fold, respectively (Fig. 7 C). Finally, several other genes identified as potential Egr3 target genes in primary myotubes (Fig. 1 D) were also up-regulated in Tg+/H muscles. *ATP1α3*, *harry*

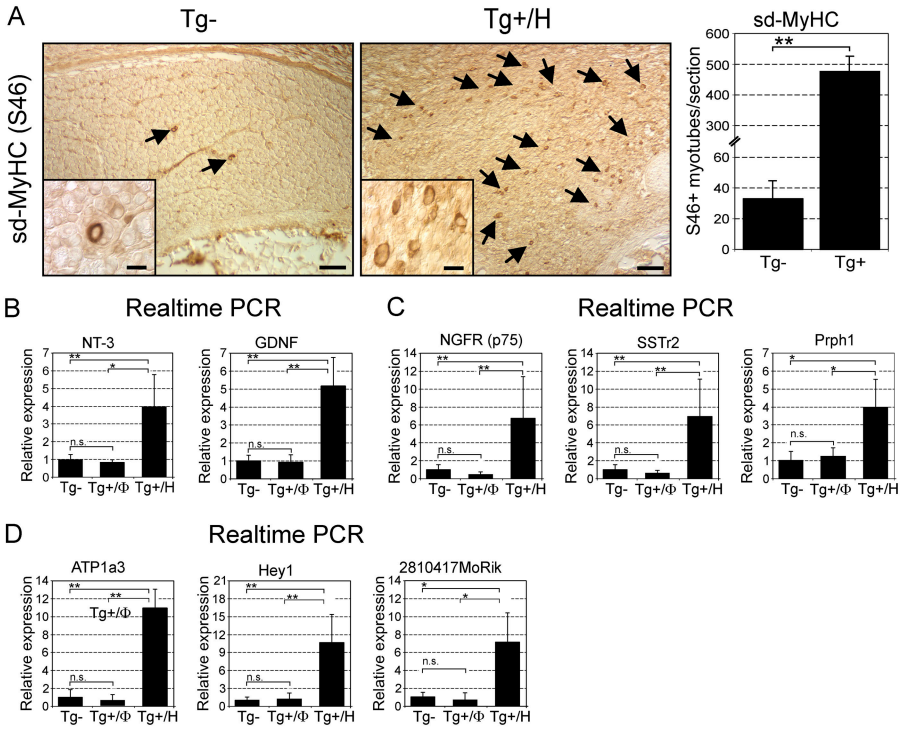

Figure 7. **Muscle fibers in HSA–*Egr3* transgenic mice express genes that are characteristic of intrafusal muscle fibers.** (A) Slow developmental myosin heavy chain (Sd-MyHC), a well-established marker for intrafusal muscle fibers, was expressed selectively by the relatively few intrafusal muscle fibers present in wild-type muscles (arrows), whereas it was markedly up-regulated by muscle fibers in Tg+/H mice. (bar graph) The number of Sd-MyHC–expressing myotubes was significantly increased in Tg+ relative to Tg− mice when the number of myotubes/section were counted in lower hindlimb muscles. Bars: 50 μm; (inset) 25 μm. (B) Similarly, expression of the neurotrophins NT-3 and GDNF, both of which are expressed in muscle primarily by intrafusal muscle fibers in E18.5 wild-type mice, were up-regulated in Tg+/H muscles but not in non-transgene-expressing muscles (Tg+/Φ) relative to wild type (Tg−). (C) The expression of NGFR (p75), SSTr2, and Prph1, three newly identified target genes that are regulated by Egr3 in spindles, were also markedly up-regulated, as were (D) several other genes (ATP1α3, Hey1, and 2810417MoRik) that were examined from the Egr3 target gene microarray analysis results (mean and standard deviation shown; $n = 4$ embryos for each genotype; *, $P < 0.05$; **, $P < 0.01$).

enhance of split 1 (*Hey1*), and *2810417MoRik* were not up-regulated in Tg+/Φ muscles relative to Tg− muscles, whereas they were up-regulated 11.2-, 10.3-, and 7.3-fold, respectively. Together, these data demonstrate that enforced Egr3 expression in skeletal myotubes leads to their transformation into muscle fibers that are structurally similar to intrafusal muscle fibers and that express many genes characteristic of them.

Discussion

During skeletal muscle development, Ia-afferents specify the fate of myotubes by engaging novel gene transcription that transforms them into biochemically and functionally distinct intrafusal muscle fibers. Recent studies have refined our understanding of this classic nerve–muscle induction process by demonstrating that Nrg1 produced by Ia-afferents and signaling through the Nrg1 tyrosine kinase receptor ErbB2 in contacted myotubes is essential to engage the spindle morphogenesis program in vivo (Andrechek et al., 2002; Hippenmeyer et al., 2002; Leu et al., 2003) and in vitro (Jacobson et al., 2004). Moreover, Nrg1 signaling from Ia-afferents is necessary to induce several transcription factors (Egr3, Pea3, ER81, and ERM), which represent at least some proximal mediators of the gene regulatory networks that are engaged by Ia-afferent contact with myotubes. We focused on the role of Egr3 in mediating particular aspects of Ia-afferent (Nrg1–ErbB2) signal transduction because our earlier work indicated that Egr3 is required for normal spindle morphogenesis (Tourtellotte and Milbrandt, 1998). Its expression is restricted to Ia-afferent–contacted myotubes, and Egr3-deficient, Ia-afferent–contacted myotubes do not acquire characteristic structural features or phenotypic markers of intrafusal muscle fibers (Tourtellotte et al., 2001; Chen et al., 2002).

Egr3 gene regulation in Ia-afferent–contacted myotubes

Egr3 regulates a relatively large network of genes within myotubes, and although not all of the genes identified were characterized further in this study, some of them were regulated by Egr3 in developing intrafusal muscle fibers. We found that NGFR (p75), SSTr2, and Prph1 are regulated by Egr3 in intrafusal muscle fibers. Of the 83 genes identified by expression analysis in myotubes, we verified that 15/15 genes tested were up-regulated, suggesting that the list of Egr3 target genes has a high degree of specificity. Moreover, the Egr3-mediated gene regulatory network is evidently complex because the target genes have tremendous functional diversity, including processes such as transcriptional regulation, intracellular signal transduction, protein processing, and cytoskeletal organization. Interestingly, however, not all genes that were up-regulated by Egr3 in myotubes in vitro were Egr3 targets in the developing intrafusal muscle fibers in vivo because neither GPR50, Rphn2, nor Arc was expressed by them. Thus, enforced expression of Egr3 in myotubes leads to target gene expression that is not necessarily restricted to the myotube–cellular context. In support of this concept, studies in progress indicate that Arc is directly regulated by Egr3 in neurons, despite the fact that it is not expressed by intrafusal muscle fibers where Egr3 is expressed at high levels (unpublished data). These results indicate that the cellular context appears to be important in providing a permissive environment for expression of a particular repertoire of Egr3 target genes that are relevant to intrafusal muscle fiber morphogenesis. Whether the target genes identified are directly regulated by Egr3 or whether they are expressed by the activation of downstream mediators of Egr3 is currently not known. Moreover, it is not known whether any of these newly identified Egr3 target genes are essential for spindle morphogenesis.

An analysis of additional Egr3 target genes by in situ hybridization will be necessary to further define the Nrg1–ErbB2–Egr3 regulatory axis that mediates intrafusal muscle fiber morphogenesis.

Ia-afferents have a broad regulatory influence over spindle morphogenesis, and Egr3 regulation is apparently only part of the complex morphogenetic signaling pathway. Many of the genes that are up-regulated during the early aspects of muscle spindle morphogenesis, such as Egr3, ERM, ER81, and Pea3, are independently regulated (Hippenmeyer et al., 2002). Thus, multiple parallel regulatory networks likely exist, and they may have distinct roles during spindle morphogenesis. For example, the role of ERM during spindle morphogenesis has not been determined because *ERM*-deficient mice die before spindle morphogenesis is initiated, but its function in muscles is not likely to be restricted to spindle morphogenesis because it is expressed by intrafusal and nonintrafusal muscle fibers. Pea3, which neither regulates Egr3 nor is regulated by Egr3, is not necessary for spindle morphogenesis despite the fact that in muscles its expression is restricted to intrafusal muscle fibers (Livet et al., 2002). Pea3, which is also expressed in motor neurons and is regulated by GDNF, has a role in patterning motor innervation rather than in spindle morphogenesis itself (Haase et al., 2002; Livet et al., 2002). Similarly, ER81 has a role in sensory patterning, which may explain the partial disruption of spindle morphogenesis in *ER81*-deficient mice secondary to altered sensory innervation on which spindle morphogenesis depends (Kucera et al., 2002). In contrast, Egr3 appears to have a selective role in mediating some aspects of Ia-afferent signaling that are related to intrafusal muscle fiber differentiation because its restricted expression to spindles is required for their normal morphogenesis. Thus, Egr3-mediated gene regulation appears to be necessary for myotubes to acquire a normal intrafusal muscle fiber phenotype.

Myotube fate specification mediated by Egr3 target gene regulation

Although Egr3 expression is necessary for normal intrafusal muscle fiber morphogenesis, it also appears to be sufficient to drive some aspects of intrafusal muscle fiber differentiation from otherwise undifferentiated skeletal myotubes. Skeletal myotubes were transformed into muscle fibers that had structural and molecular similarities to intrafusal muscle fibers when Egr3 expression was enforced independently of Ia-afferent signaling. Moreover, several established intrafusal muscle fiber markers, including Sd-MyHC, NT-3, and GDNF were up-regulated in HSA–*Egr3* transgene-expressing muscles. Similarly, three new Egr3 target genes that were selectively expressed by intrafusal muscle fibers (*NGFR [p75]*, *SSTr2*, and *Prph1*) and three additional Egr3 target genes (*ATP1α3*, *Hey1*, and *2810417MoRik*) were all up-regulated in transgene-expressing muscles. However, the transformed muscle fibers did not acquire the motor or sensory innervation that is characteristic of normal intrafusal muscle fibers, suggesting that Egr3 has a role that is distinct from other Ia-afferent–mediated pathways that may be required to establish and/or maintain these interactions. Moreover, the transformed myotubes did not acquire fusiform capsules that may normally be derived from terminal Schwann cells of the innervating sensory axons. Finally, somatic motor neurons were almost completely absent in HSA–*Egr3* transgenic mice, indicating that the transformed muscle fibers were not capable of sustaining skeletomotor innervation that would have been present had they not expressed Egr3 and differentiated into intrafusal muscle fibers. It will be necessary to conditionally express Egr3 in skeletal myotubes in order to study the fate of motor and sensory innervation during development because the transgenic founders died and could be generated only in small numbers.

Sensory control over the morphogenesis of many mechanoreceptors is recognized as a common theme in their ontogeny. A detailed understanding of the gene regulatory mechanisms that are governed by sensory axon innervation of muscle spindles may be applicable to mechanisms used during the ontogeny of other sensory mechanoreceptors. These studies define a set of Egr3-regulated target genes, some of which appear to be relevant to intrafusal muscle fiber morphogenesis. Egr3 is regulated by Nrg1–ErbB2 signaling, and it appears to mediate gene expression that is necessary and sufficient to specify the fate of Ia-afferent–contacted myotubes to become intrafusal muscle fibers and to mediate their morphogenesis.

Materials and methods

Animals and preparation of tissues

Egr3-deficient mice were mated on a C57BL/6/129SvJ background and were generated and genotyped as previously described (Tourtellotte and Milbrandt, 1998; Whitehead et al., 2005).

The HSA–*Egr3* transgenic mice were generated by using the previously characterized HSA promoter to express Egr3 in skeletal myotubes (Muscat et al., 1992). The transgenic construct was derived from pBSX-HSAvpA (Crawford et al., 2000; provided by J. Chamberlain, University of Washington, Seattle, WA) by cloning a full-length, HA-tagged Egr3 cDNA into the NotI site of pBSX-HSAvpA. The construct was injected into B6SJL embryo pronuclei and transplanted into recipient females using standard procedures (provided by L. Doglio and R. Alvarez, Northwestern University, Chicago, IL). The transgene-expressing founder mice were not postnatally viable, and, therefore, recipient females were killed to analyze founder embryos at various gestational ages. Each embryo was genotyped using primers 5′-AAACTCGCCGAGAAGCTGCCGGTG-3′ (sense) and 5′-TGTGCATCCATCGCTAGCTCGCTC-3′ (antisense) to amplify a 230-bp fragment of the endogenous Egr3 genomic locus. A third primer, 5′-TCAGGGAGACGTGGAGGCCATGTA-3′, was used to amplify a 413-bp fragment of the Egr3 cDNA transgene (nonintron containing) when paired with the sense probe in a multiplex PCR reaction.

The day of vaginal plug for all of the mice was considered as E0.5. The embryos were isolated by cesarean section, were rapidly decapitated, were fixed at 4°C in 100 mM phosphate-buffered 4% PFA, and were either cryoprotected for 24 h in 30% phosphate-buffered sucrose and frozen or were directly embedded in paraffin. Serial paraffin sections (8 μm) or frozen sections (15 μm) were generated. Some tissues were embedded in resin, in which case the fixative was substituted for 2.4% glutaraldehyde and 1% PFA. The tissues were dehydrated in increasing concentrations of ethanol that was infiltrated with Embed 812 resin (Electron Microscopy Sciences) and heat cured at 60°C overnight. Resin sections were cut at 1 μm and were counterstained with toluidine blue (Sigma-Aldrich).

Myoblast isolation and myotube culture

Hindlimb skeletal muscle tissues were isolated from wild-type, postnatal (3–5) mice. Myoblasts were isolated by enzymatically dissociating (dispase, grade II, 2.4 U/ml; Boehringer), and the muscle tissues were triturated according to previously published procedures (Rando and Blau, 1994). Myoblasts were purified by multiple rounds of preplating on collagen-coated tissue culture plates (0.01% type I; Sigma-Aldrich) and expanded in growth medium (Ham's F10; Mediatech), 20% FBS, and 2.5 ng/ml basic FGF (Promega). Purified primary myoblasts were plated onto collagen-coated plates and were differentiated into multinucleated myotubes for 10 d using differentiation medium (Ham's F10 and 5% horse serum).

Recombinant adenovirus preparation, characterization, and myotube infection

Recombinant adenoviruses were generated using homologous recombination in *Escherichia coli* as previously described (He et al., 1998). To generate an adenovirus that expressed transcriptionally active Egr3, the full-length rat Egr3 cDNA (GenBank/EMBL/DDBJ accession no. NP058782, aa 1–387) was cloned into the BglII site of pAdTrackCMV. Similarly, a transcriptionally inactive, COOH-terminal truncation of Egr3 that lacked the entire three zinc finger DNA-binding and COOH-terminal domains (GenBank NP058782, aa 1–245) was cloned into the BglII site of pAdTrackCMV. Each recombinant pAdTrack shuttle vector, including pAdTrackCMV without a cDNA insert, was recombined by homologous recombination into the adenoviral genomic plasmid (pAdEasy) in BJ5183 *E. coli* to generate a recombinant, replication-deficient adenoviral genome plasmid. The three replication-deficient viruses (EGFP, $Egr3_{WT}$, and $Egr3_{Tr}$) were packaged and amplified in transfected HEK-293 cells, purified, concentrated on cesium chloride gradients, and titered using EGFP fluorescence and TCID50.

NIH-3T3 fibroblasts were infected with the recombinant adenoviruses at a multiplicity of infection (MOI) of 10–20 to obtain ~70–80% infection efficiency. Total cell lysates were obtained 24 h after infection, and a Western blot using an NH_2-terminal Egr3, rabbit polyclonal antibody (O'Donovan et al., 1998) demonstrated a high expression of full-length and truncated Egr3 protein as expected. Myoblasts were cotransfected with an early growth response element (ERE) containing a firefly luciferase reporter plasmid (Swirnoff and Milbrandt, 1995) and a Renilla luciferase reporter plasmid (pRL-TK; Promega) for transfection efficiency control. 16 h after plasmid cotransfection, the cells were infected with either control EGFP, $Egr3_{WT}$, or $Egr3_{Tr}$ adenoviruses at an MOI of 10 to obtain 80–90% infection efficiency. 8 h after infection, a dual-reporter luciferase assay was performed to quantify ERE activation by the virally produced proteins according to the manufacturer's specifications (Promega).

Primary myotubes that differentiated for 10 d in vitro were infected with either $Egr3_{WT}$ or $Egr3_{Tr}$ adenovirus at an MOI of 100 to obtain 100% infection efficiency. Although myotubes were relatively resistant to infection and required a relatively high MOI for optimal infection, no evidence of toxicity was noted 24 h after infection at the time that total RNA was extracted from the infected myotubes.

Gene expression profiling

5 μg of total RNA was converted to cDNA using the superscript reverse transcriptase (Invitrogen) and the T7-Oligo (dT) promoter primer kit (Affymetrix, Inc.). The cDNA was purified using the GeneChip sample cleanup module (Affymetrix, Inc.) and was used for the in vitro synthesis of biotin-labeled cRNA using the GeneChip expression 3′-amplification reagents for IVT labeling (Affymetrix, Inc.) at 37°C for 16 h. cRNA was fragmented into 35–200–bp fragments using a magnesium acetate buffer (Affymetrix, Inc.). 10 μg of labeled cRNA were hybridized to GeneChip mouse expression arrays (430A and 430B; Affymetrix, Inc.) for 16 h at 45°C. The GeneChips were washed and stained according to the manufacturer's recommendations (Affymetrix, Inc.) using the GeneChips fluidics station (model 450; Affymetrix, Inc.). This procedure included washing the chips with phycoerythrin-streptavidin, performing signal amplification by a second staining with biotinalyted antistreptavidin, and performing a third staining with phycoerythrin-streptavidin. Each chip was scanned using the GeneChips scanner (model 3000; Affymetrix, Inc.). Signal intensity and detection calls were generated using the GeneChip operating software (Affymetrix, Inc.). The absolute intensity values of each chip were scaled to the same target intensity value of 150 in order to normalize the data for inter-array comparisons. Four chip comparisons were generated using the samples from transcriptionally inactive Egr3 ($Egr3_{Tr}$)-infected myotubes as the baseline. A list of up-regulated and down-regulated genes with a greater than twofold change was generated using an iterative comparison analysis (Chen et al., 2000). This method has been shown by the National Institutes of Health's (NIH) Microarray Consortium to increase specificity (>80% true positive rates) in the resultant gene list. The annotated MAGE-ML and image fields are available at http://arrayconsortium.tgen.org.

Real-time PCR

Total RNA was isolated from the primary myotubes or mouse muscle tissue using TRIzol (Invitrogen), and 0.5–1.0 μg was reverse transcribed using random octomer priming and powerscript reverse transcriptase according to the manufacturer's specifications (BD Biosciences). Real-time PCR was performed on a sequence detector (model SDS5700; Applied Biosystems) using Syber green fluorescence chemistry (Molecular Probes). Non–intron-spanning primers that amplified the coding sequence were designed for each target gene. For each primer pair, melt curves were generated to identify the optimal temperature to quantify the total fluorescence after each amplification cycle so that only the gene-specific amplicon fluorescence was measured during the PCR reaction. For each primer pair, serial dilutions of mouse genomic DNA were used to generate a standard curve, to characterize their linear dynamic range, and to provide relative DNA concentrations that correlated with gene expression at particular threshold cycle numbers in the linear range of amplification. For each cDNA sample, the relative level of the target gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was interpolated from the gene-specific standard curves. The expression of each target gene was normalized to the level of GAPDH expression, and the normalized expression values were again normalized to a reference condition, such as gene expression in nontransgenic muscle or $Egr3_{Tr}$-infected myotubes, to assess fold change in expression that is relative to that condition.

Non–intron-spanning primer sequences were used for real-time PCR analysis. They amplified the following: *Egr3* (GenBank NM_018781.1, nt 163–563), *GAPDH* (GenBank NM_001001303, nt 210–635), *NGFR/p75* (GenBank BC038365, nt 1123–1369), *SSTr2* (GenBank NM_009217, nt 603–859), *Prph1* (GenBank NM_013639, nt 109–616), *ATP1α3* (GenBank NM_144921, nt 3074–3293), *Hey1* (GenBank NM_010423, nt 669–670), *2810417MoRik* (GenBank NM_026516, nt 910–1209), *Sbsn* (GenBank NM_172205, nt 36–333), *Arc* (GenBank NM_018790, nt 1175–1483), *GPR50* (GenBank AF065145, nt 256–567), *Rhpn2* (GenBank NM_011872, nt 1821–2077), *KLK7* (GenBank NM_011872, nt 94–396), *Krtdap* (GenBank AB011028, nt 8–297), *Ntf3/NT-3* (GenBank NM_008742, nt 233–633), *GDNF* (GenBank D88351, nt 2552–2769), *SNAP25* (GenBank NM_011428, nt 191–498), *Ube3a* (GenBank NM_173010, nt 964–1264), *Clcn2* (GenBank NM_009900, nt 2710–3009), and *Neud4* (GenBank NM_013874, nt 1327–1634).

In situ hybridization and immunohistochemistry

In situ hybridization was performed using digoxygenin-labeled riboprobes and previously published standard protocols (Darby, 2000). In situ hybridization probes were generated to label coding regions from the published mRNA sequences. The PCR-generated amplicons were polished (End-it; Epicentre), were subcloned into the EcoRV site of Bluescript (Stratagene), and were sequence verified. Sense and antisense riboprobes were synthesized using in vitro transcription and digoxygenin-labeled dUTP. In all cases, the sense probe was used on parallel tissue sections as a control for nonspecific labeling, and antisense probes were used to examine gene-specific expression.

The probes used for in situ hybridization spanned the following coding sequences: *Egr3* (GenBank NM018781, nt 890–1389), *Pea3* (GenBank NM_008815, nt 533–1082), *GPR50* (GenBank, AF065145, nt 157–656), *Rhpn2* (GenBank NM_027897, nt 1381–1890), *Arc* (GenBank NM_018790, 577–1082), *NGFR* (*p75*) (GenBank BC038365, nt 253–767), *SSTr2* (GenBank NM_009217, nt 685–1185), and *Prph1* (GenBank NM_013639, nt 109–616). Wild-type muscle spindles were identified by their characteristic morphological appearance (spindle capsules surrounding muscle fibers with internal nuclei). In *Egr3*-deficient mice, prospective "spindles" were identified by Pea3 expression, which marks Ia-afferent–contacted myotubes and intrafusal muscle fibers.

Immunoperoxidase histochemistry was performed using specific rabbit polyclonal antibodies that cross-reacted with the COOH-terminal region of Egr3 (sc-190; Santa Cruz Biotechnology, Inc.), the NH_2-terminal region of Egr3 (O'Donovan et al., 1998; provided by J. Baraban, John Hopkins University, Baltimore, MD), Pv (R301; provided by K. Baimbridge, University of British Columbia, Canada), ATP1α3 (Upstate Biotechnology), and Sd-MyHC (S46; provided by D. Fischman, Cornell University, New York, NY). A biotinylated, antirabbit secondary antibody (Jackson ImmunoResearch Laboratories) and avidin–biotin complex histochemistry using diaminobenzidine as a chromagen was performed according to the manufacturer's specifications (Vector Laboratories). In some cases, an antirabbit, Cy3-conjugated secondary antibody was substituted (Jackson ImmunoResearch Laboratories). All photographs were acquired with a digital camera (model RT-Slider; Spot) attached to a microscope (model E600; Nikon), and the images were processed using Adobe Photoshop software.

Neuron counts

Stereological quantification of the total number of Pv+ neurons within the fifth lumbar DRG ($n = 4$ per genotype) was performed using the optical dissector method (StereoInvestigator, Microbrightfield). Every fourth section through the fifth lumbar DRG was analyzed. Contours containing a single DRG were optically sectioned using a 100× objective (NA 1.4) and an oil substage condenser. The sampling sites were 175 × 100 μm,

and the counting frame boundaries were 80 × 60 × 6 μm in height. Only immunopositive neurons with sharply focused nuclei (containing at least one nucleolus) within the optical dissector counting frame boundaries were tallied. The total number of Pv+ neurons per DRG was estimated by the StereoInvestigator software.

Statistical measures

The Kolmogorov-Smirnov test was used to detect departures of the data from normality. F-tests were used to check for equal variances, and, in the case of unequal variances, the nonparametric Mann-Whitney U test was used. When variances were equal, the *t* test was used. The acceptance of significance was set to $P < 0.05$.

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