Molecular Mechanisms of Neuronal Circuit Assembly in the Vertebrate Spinal Cord

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Simon Hippenmeyer

aus Gottlieben / TG und Pratteln / BL

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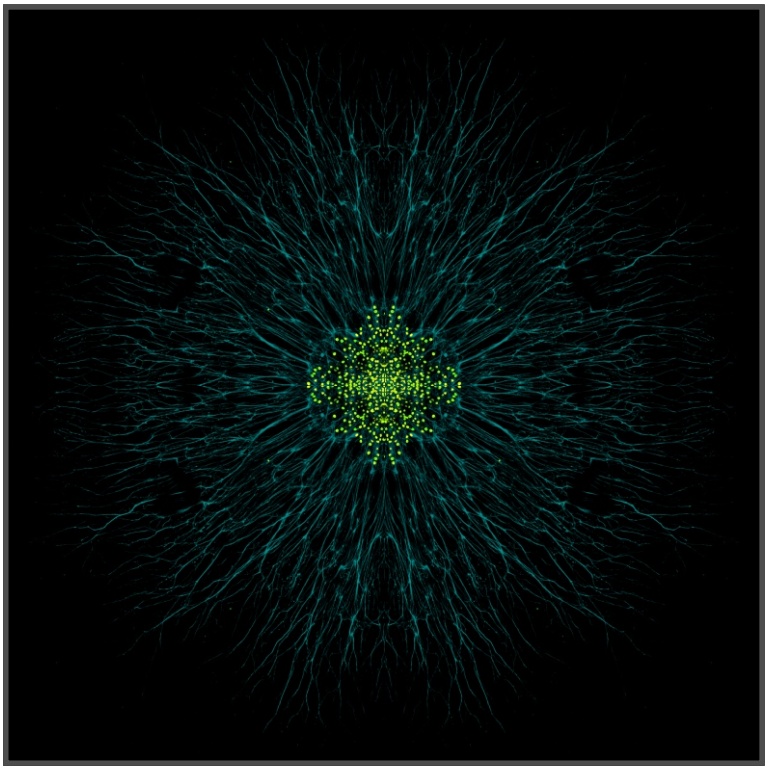

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Ausgeführt unter der Leitung von Prof. Dr. S. Arber

Abteilung Zellbiologie
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und

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Basel, Juni 2004

to

Mum & Dad

Acknowledgments

In the first place I would like to thank Silvia Arber for the exciting and excellent scientific environment that I could enjoy during my PhD studies in her lab. I thank you Silvia, for always having time to discuss scientific and personal issues and for giving me great support throughout my whole dissertation period. I very much appreciate that you provided me with various enormous interesting and stimulating opportunities to meet the scientific community all around the world where I also got a lot of inspiration and motivation for the every day lab business.

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Preface

The contents of the following dissertation include nine chapters. Chapter one to four serve as an introduction into the field of developmental neurobiology with particular emphasis on molecular cell extrinsic and intrinsic signaling pathways involved in the assembly of neuronal circuits as well as the neuronal system and molecular regulators that were in the focus throughout the whole thesis. The four chapters five to eight are based exclusively upon experimental work. Each of these chapters describes and discusses new findings obtained in the course of my dissertation. The thesis closes with a final chapter dedicated to a critical discussion of open issues and future perspectives.

A general short introduction into the field that is followed by a paragraph displaying the specific aim and topic of the thesis is presented in chapter one. In chapter two, recent findings are recapitulated with respect to extrinsic target derived signaling molecules that act retrogradely on the cell bodies of neurons to shape and consolidate terminal maturation of specific subpopulations of interconnected neurons. Molecular mechanisms including various cell intrinsic properties and extrinsic signaling aspects involved in the development of the monosynaptic stretch reflex circuit are discussed in detail in chapter three. Two transcription factors of the ETS class have been described to fulfill essential roles in the assembly of the monosynaptic circuit. The general molecular features that characterize the ETS family of transcription factors are outlined in some detail within chapter four. Chapter five, six, seven, and eight represent original research contributions on the basis of various experimental approaches. Chapter five describes a role for a specific signaling molecule, Neuregulin1, in muscle spindle differentiation. Chapter six and seven deal with the requirement of temporally controlled transcriptional programs in the course of differentiation of populations of sensory and motor neurons. Chapter eight addresses the specificity of ETS transcription factors in the establishment of sensory-motor connections in the spinal cord. The last chapter nine represents a final critical discussion with respect to open issues and future perspectives.

Several chapters of this dissertation include parts that have been subject to submission for and/or final publication in the form of reviews or original research articles. I would like to take the opportunity to thank and honor all the collaborators and co-authors that contributed in one or the other way to these publications. In addition and most importantly I would like to thank the following people for providing mutant mouse strains that served as a basis and extremely valuable reagents for various experimental analyses described throughout the experimental studies: C. Birchmeier and S.J. Burden (*Nrg1-ΔEGF-LacZ* and *Nrg1-flox*); L. Role (*CRD-Nrg1*); L. Parada

(*TrkC*); D.J. Anderson (*Ngn1*); W. Tourtelotte (*Egr3*); U. Müller (*ErbB2-flox* and *HSA-Cre*); T.M. Jessell and S. Arber (*Isl1-Cre*, *Hb9-NLZ*, *Hb9-Cre*, *Er81-NLZ*, *Er81-Pea3*, *Er81-EWS-Pea3*); S. Arber, P. Caroni, and M. Sigrist (*Thy1-spGFP*, *Tau-mGFP* and *PV-Cre*); Furthermore, some figure panels (that were included in publications) within this dissertation show primary original research data that were acquired by Silvia Arber (Figure 13A, B, D-G; Figure 44C, D) Neil Shneider (Figure 23B-D, F-H), Thomas Portmann (Figure 27A) and Eline Vrieseling (Figure 28E-H). The generous sharing of these individual data sets is highly appreciated and acknowledged. I am confident that inclusion of these data allows the presentation of the respective chapters in a more consistent way.

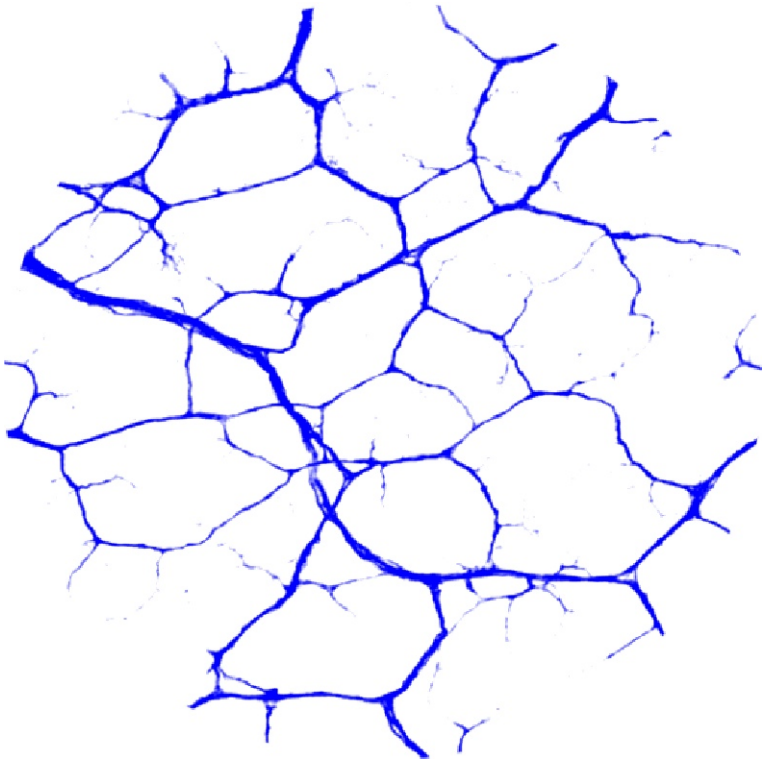
I thank the reader for interest in this dissertation and hope you will appreciate the reading.

CONTENTS IN BRIEF

CONTENTS

Chapter 1

Introduction
-
Assembly of Neuronal Circuits



Get Connected

Understanding the biological mechanisms involved in shaping our mind - how we perceive the world individually, remember and recall perception from memory, think and how emotions color our thinking, act and react in a conscious way - is ambitious but represents the ultimate goal of neural science.

The human brain is a sophisticated neuronal matrix composed of more than 100 billion individual nerve cells. These neurons are interconnected in very complex neuronal circuits. The precision with which these circuits are assembled is crucial and largely responsible for all brain functions and thus our thoughts and actions. Diverse specialized functions of the vertebrate nervous system range from sensory perception and motor coordination to motivation and memory. Appropriate performance of these various functions depends on selective synaptic connections formed between distinct subpopulations of neurons. These specific connections linking different neurons within a neuronal circuit are established successively during development and represent the underlying basis for the formation of defined mature neuronal circuits.

Functional neuronal circuits are assembled in a series of developmental steps (Albright et al., 2000; Jessell and Sanes, 2000). An essential prerequisite for this assembly process is the ‘generation’ of neurons involving processes such as neural induction and neurogenesis. Subsequently, distinct newly born nerve cells are being specified and become determined cell-intrinsically. Directed outgrowth and extension of axons towards future distant targets and correct choice of specific termination zones within target regions is required to achieve appropriate connectivity between neurons whose cell bodies are often found at long distances. Finally the actual process of synaptogenesis paired with refinement mechanisms allow the formation of selective and specific synaptic contacts between appropriate neuronal partners.

At the molecular level, efforts especially over the last three decades have resulted in the identification of a rich catalog of molecules and genes with evolutionary conserved functional roles during the development of the nervous system and the progressive steps involved in the assembly of neuronal circuits.

The combinatorial involvement of numerous families of molecules paired with the extraordinary spatial complexity of interconnected neuronal circuits demands however for experimental systems that are sufficiently defined on both the structural and physiological level, to study the specific role

of particular molecules in the various developmental steps involved in the assembly of a mature and functional neuronal circuit.

The reflex circuits in the spinal cord represent a suitable neuronal system, which has been studied extensively at the level of both structural circuit organization and physiological function. The most studied spinal reflex circuit is the monosynaptic stretch reflex circuit that represents the simplest and best understood neuronal circuit within the vertebrate nervous system (Sherrington, 1910; Eccles et al., 1957; Brown, 1981; Glover, 2000).

Connectivity in the Spinal Cord

Charles Sherrington was among the first pioneers who recognized the importance of specific sensory input in the regulation of coordinated movements. As early as 1906 he proposed that stereotyped movements in response to activation of sensory receptors in the peripheral muscles (termed reflexes), serve as the basic units for movement (Sherrington, 1906). More specifically, the monosynaptic stretch reflex circuit is responsible for the control of stereotyped sensory-motor behavior (Eccles et al., 1957; Brown, 1981). A major purposeful action of spinal reflexes therefore is required for proprioception (from *propria*, 'my own' or 'self'; internal representation or sense of static position and changes in body movement). A common 'functionality test' for the stretch reflex circuit represents the tapping on the patellar tendon resulting in the extension of the lower leg. The stretch reflex involves the interplay of a sensory and a motor unit and leads to a contraction of muscle as a consequence to changes (stretch) in the length and/or tension of the respective muscle. The sensory and motor units are interconnected through proprioceptive afferent neurons that transmit peripheral sensory information about the state of muscle contraction to the central nervous system (Eccles et al., 1957; Brown, 1981).

The motor unit includes α-motor neurons and the corresponding innervated extrafusal muscle fibers. The cell bodies of the α-motor neurons, innervating extrafusal muscle fibers at the neuromuscular junction (NMJ) of a particular muscle, are clustered into a so-called motor neuron pool located within the ventral spinal cord (Landmesser, 2001).

Proprioceptive sensory neurons are neural crest derivatives (Knecht and Bronner-Fraser, 2002). Their cell bodies are located within dorsal root ganglia (DRG) on either side of the spinal cord at each segmental level. All DRG sensory neurons extend a peripheral axon and central axonal branch

invading the spinal cord (Brown, 1981). Within proprioceptive sensory neurons, group Ia afferents innervate muscle spindles, small encapsulated spindle-like or fusiform shaped sensory receptors that are sensitive to a change in muscle length and are embedded in parallel within extrafusal muscle fibers (Figure 1; Zelena, 1994, Maier, 1997). In contrast, group Ib afferents innervate Golgi tendon organs (GTOs), located in series at the junction between muscle fibers and tendon and are sensitive to changes in muscle tension (Figure 1; Zelena and Soukup, 1977).

In the spinal cord, both Ia and Ib afferents establish a termination zone and synaptic connections with interneurons in the intermediate spinal cord but only Ia afferents form direct monosynaptic connections with α-motor neurons (Figure 1; Brown, 1981; Eccles et al., 1957; Frank and Wenner, 1993; Glover, 2000).

Ia afferents from a particular muscle do not only excite α-motor neurons innervating the same muscle (homonymous connections) but also those innervating muscles with a similar mechanical action (heteronymous connections). In contrast, 'antagonistic' α-motor neurons do not get direct excitatory input from Ia afferents but rather receive indirect inhibition through Ia afferents making connections to inhibitory interneurons (Figure 2; Frank and Wenner, 1993; Wenner and Frank, 1995; Glover, 2000). This specificity in connectivity is absolutely essential for the appropriate transmission of a sensory input to a motor unit controlling temporally and spatially adequate contraction of particular muscles. In summary, two classes of neurons, α-motor neurons and Ia afferents, form a simple functional neuronal circuit via interconnection by a single (mono) synapse that contributes to appropriate movement and thereby motor behavior.

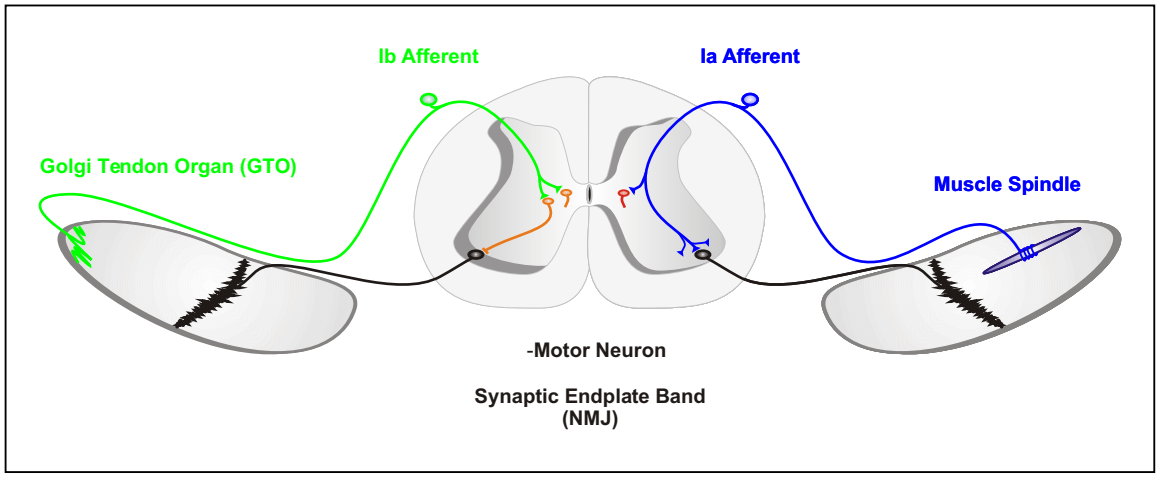


Figure 1. Peripheral and Central Projections of Ia and Ib Proprioceptive Sensory Afferents. Left: Group Ib muscle afferents (green) form peripheral associations with Golgi tendon organs from which they receive peripheral sensory input. Centrally, Ib afferents project to the intermediate spinal cord where synaptic contacts are made with interneurons (orange) inhibiting -motor neurons (black). Right: Group Ia muscle afferents (blue) innervate muscle spindles in the periphery. Centrally, Ia afferents establish two major termination zones where in the intermediate spinal cord synapses are made with interneurons (red) and within the ventral termination zone direct monosynaptic excitatory connections are formed with -motor neurons. -motor neurons innervate extrafusal muscle fibers at the neuromuscular junction (NMJ) within the synaptic endplate band (black) of a particular muscle.

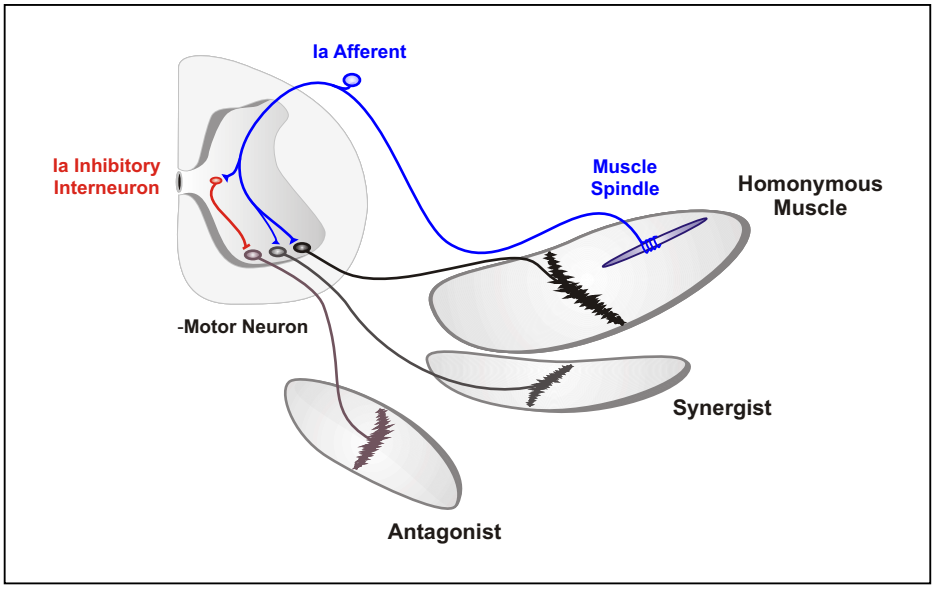


Figure 2. Selective Ia Afferent - -Motor Neuron Connectivity in the Spinal Cord. Ia afferents (blue) connected to peripheral muscle spindles make excitatory connections on -motor neurons (black) that innervate the same (homonymous) muscle from which they arise and on -motor neurons (grey) that innervate synergist muscles. Ia afferents also act through Ia inhibitory interneurons (red) to inhibit -motor neurons (purple) that innervate antagonist muscles.

Genetic Control of Specificity in Sensory-Motor Connectivity

Genetic determination plays a fundamental role in the development of selective monosynaptic connections indicating that appropriate development and assembly of the monosynaptic stretch reflex circuit is 'hard-wired' (neuronal circuits that from during embryonic development without obvious need for activity or experience) to a large extent. Specific molecular cell intrinsic and extrinsic mechanisms appear to complement each other and represent the integral part of the essential genetic basis to achieve the selective formation of functional synaptic connections within the monosynaptic reflex circuit in the spinal cord (Chen and Frank, 1999; Glover, 2000).

During development and establishment of specific monosynaptic connections, proprioceptive Ia afferents and α-motor neurons become progressively specified. During this process the earliest stages of differentiation are controlled mostly by cell intrinsic properties acquired or inherited at progenitor cell stages. However, during later phases, peripheral signals encountered by axonal growth cones are essential for terminal aspects of Ia afferent differentiation (Edlund and Jessell, 1999; Livet et al., 2002; Haase et al., 2002; Patel et al., 2003). Once sensory neurons have acquired a Ia afferent fate, they can also be distinguished by their specific patterns of connections with α-motor neurons (Frank and Wenner, 1993). Ia afferent connections to motor neurons exhibit appropriate specificity from the time they are first established and mechanisms such as activity-dependent remodeling do not play a role in the formation of specific synaptic connections between Ia afferents and α-motor neurons (Frank and Jackson, 1986; Mendelson and Frank, 1991). Ia afferents selectively innervate appropriate α-motor neurons even when inappropriate pools of α-motor neurons are in close proximity. At the stage when Ia afferent axons begin to innervate a particular muscle, specific signals that are encountered within the muscle appear to dictate the selection of target α-motor neurons in the spinal cord by central Ia afferent projections by specification through a peripheral signal (Frank and Wenner, 1993; Wenner and Frank, 1995). Thus, central monosynaptic connections always maintain stretch reflex specificity.

The molecular mechanisms by which Ia afferents are being specified peripherally are only beginning to be revealed. A first example of an identified uniform retrograde signal that plays a role in actively instructing Ia afferent cell bodies is the neurotrophin NT-3 (Patel et al., 2003). Growth cones of Ia afferents, that encounter target derived NT-3, tell (signal retrogradely to) the cell bodies to upregulate the ETS transcription factor Er81 (Sharrocks, 2001) that is in turn involved in promoting the establishment of the Ia afferent ventral termination zone in the spinal

cord (Arber et al., 2000; see chapter two for detailed discussion of this emerging retrograde peripheral signaling principle).

In summary, Ia afferents are actively instructed by peripheral signals both for establishment of projections as well as selectivity of connections to α-motor neurons in the spinal cord. Moreover, electrical activity or experience is not required to establish the Ia afferent ventral termination zone and selective synapses onto α-motor neurons in the spinal cord. Together, cell intrinsic and extrinsic genetic mechanisms appear to have a critical impact in the process of synaptic inter-connection of sensory- and motor neurons within the stretch reflex circuit.

In addition, with respect to muscle spindle development it is interesting to note that, despite the low number, in comparison to extrafusal muscle fibers, this number as well as the arrangement and pattern of the intrafusal fibers is highly conserved between different individuals but very distinct in different muscles (Zelena, 1994; Maier, 1997). Furthermore, sensory muscle innervation results in a precise coincidence of Ia afferent terminals with the pattern of muscle spindles within distinct muscles. Thus it becomes apparent that Ia afferents might have an instructive role during development of muscle spindles, representing a crucial component of the sensory unit of the monosynaptic stretch reflex circuit (Zelena, 1994; Maier 1997)

While it is clear that various genetic mechanisms appear to be involved in the development and specification of the elements that are assembled coordinately into the functional monosynaptic stretch reflex circuit, the actual molecular signaling cascades involved are mostly unknown. Gene expression patterns provide a starting point and potential candidate molecules that might be involved in the assembly of the spinal monosynaptic reflex circuit. However, only very few proteins have been implicated and functionally associated in controlling differentiation and assembly processes of the monosynaptic reflex circuit in the spinal cord. Nevertheless, integrated genetic, biochemical, cell biological and electrophysiological approaches dedicated to functional analysis of these expressed candidate molecules should contribute to our knowledge of how neuronal circuits are assembled during development. Using this information, directed genetic and/or molecular manipulation of defined neuronal systems might allow further insight into the generation, processing and transmission of circuit specific neuronal information among diverse behavior paradigms.

Topic and Aim of this Dissertation

Development of the monosynaptic stretch reflex circuit in the spinal cord is genetically preprogrammed. In the course of proprioceptive Ia afferent specification and differentiation several distinct transcriptional signaling programs direct appropriate cell fate acquisition. Adequate endowment of Ia afferents with specialized molecular features in response to both cell-intrinsic and extrinsic signaling events appears to be a crucial requirement for the inter-connection of sensory with motor units within the spinal monosynaptic stretch reflex circuit.

A recurrent intention throughout this thesis was the functional analysis of distinct molecular mechanisms and components of different signaling cascades that play essential roles in the assembly of the spinal monosynaptic stretch reflex circuit within the vertebrate central nervous system.

More specifically, a long-standing hypothesis was addressed in which a signaling interaction originating from Ia afferents to prospective intrafusal muscle fibers has been proposed to positively influence initial specification and/or maintenance of muscle spindles (Maier, 1997). In particular, the role of the signaling molecule Neuregulin-1 (Nrg1) in muscle spindle differentiation was analyzed in detail. The Nrg1 signaling pathway has been shown to be required in a number of related cellular paradigms (Burden and Yarden, 1997; Volk, 1999; Garratt et al., 2000; Buonanno and Fischbach, 2001). Therefore it was hypothesized that Nrg1 signaling might fulfill analogous roles during the process of muscle spindle induction.

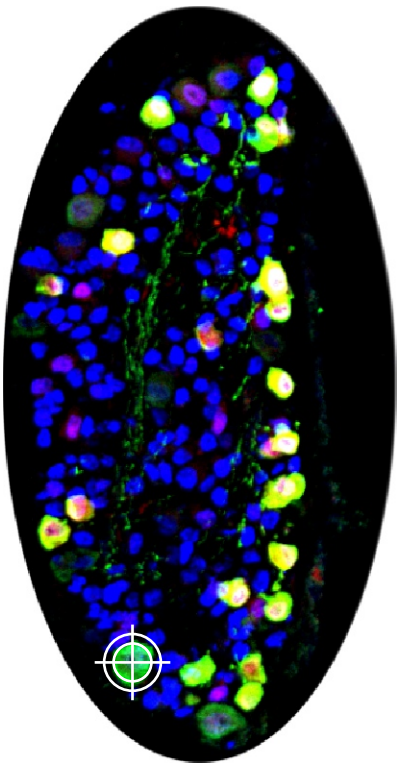
A further major theme of my thesis was centered on an *in vivo* structure-function analysis of the Pea3 subfamily of ETS transcription factors including Pea3, Er81, and Erm, respectively (Sharrocks, 2001). A critical role for Er81 in the establishment of the ventral Ia afferent termination zone in the spinal cord has recently been described (Arber et al., 2000). In addition, Er81 expression in Ia afferents is under control of peripheral signals and its expression is only induced about two to three days after Ia afferents exit the cell cycle when their axons are in close proximity with their targets (Lin et al., 1998; Patel et al., 2003). The significance and importance of this ‘delay’ was addressed in detail by temporal manipulation of induction of ETS transcriptional activity.

Chapter 2

Control of Neuronal Phenotype
-
What Targets Tell the Cell Bodies

S. Hippenmeyer, I. Kramer* and S. Arber*

Trends in Neurosciences
2004 (in press)



Summary

The assembly of neuronal circuits is controlled by the sequential acquisition of neuronal subpopulation specific identities at progressive developmental steps. Whereas neuronal features involved in initial phases of differentiation are already established at cell cycle exit, recent findings mainly based on work in the peripheral nervous system suggest that the timely integration of signals encountered *en route* to the targets and from the target region itself is essential to control late steps in connectivity. As neurons project towards their targets they require target-derived signals to establish mature axonal projections and acquire neuronal traits such as the expression of distinct combinations of neurotransmitters. Recent evidence presented in this review shows that this principle of a signaling interplay between target-derived signals and neuronal cell bodies is often mediated through transcriptional events and is evolutionary conserved.

Introduction

The assembly of neuronal circuits is controlled by highly stereotyped and genetically encoded developmental programs to ensure appropriate neuronal subtype specification and precision of synaptic connectivity in the mature nervous system. The first steps towards neuronal subtype specification are initiated at stages before neural progenitors generate postmitotic neurons when defined transcriptional programs are established in response to local signaling sources patterning the nervous system (Edlund and Jessell, 1999; Puelles and Rubenstein, 2003). Early postmitotic neurons thus inherit a distinct intrinsic fate reflecting their progenitor cell identity (Edlund and Jessell, 1999) and temporal birth order (Pearson and Doe, 2003; Hanashima et al., 2004). Early steps in axon pathfinding towards the target region and the initiation of the elaboration of dendrites are thought to rely on properties that represent these early postmitotic fates of particular neuronal subpopulations (Jessell, 2000; Shirasaki and Pfaff, 2002; Bertrand et al., 2002).

As axons extend their growth cones towards the target region, they encounter a variety of axon guidance cues along their paths that have to be interpreted and integrated. Many of the downstream responses occur locally at a rapid time scale and translate into cytoskeletal changes allowing the growth cone to navigate correctly towards its destined target area (Dickson, 2002; Huber et al., 2003). These local responses depend on the receptors and signaling molecules present at the growth cone and can lead to different responses in neuronal subpopulations endowed with a different complement of expressed genes (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001). Not all signals impinging on growth cones, however, are integrated locally but some of them fulfill

their role in neuronal differentiation by acting retrogradely on the cell body. A well established role for target-derived signals which has been the focus of many past studies is the control of neuronal survival due to the presence of limiting amounts of neurotrophic factors (Bibel and Barde, 2000; Ginty and Segal, 2002; Campenot and MacInnis, 2004). Recent studies have provided evidence that not only neuronal survival but also the acquisition of cellular identity and neuronal circuit assembly can be mediated through retrograde signals encountered *en route* to the target or from the target region itself. Several of these studies have begun to reveal such other biological functions of neurotrophic factors by mutations in key regulators of apoptosis to prevent cell death. This review will focus on recent studies with a particular emphasis on neural systems in which some of the molecular components mediating changes in neuronal phenotype in response to target-derived cues have been identified.

The Role of Target-derived Factors in the Differentiation of Sympathetic Neurons

Sympathetic ganglia of the autonomic nervous system are neural crest derived structures, which form during embryonic development through successive steps of differentiation. Sympathogenic neural crest cells emanate from the dorsal neural tube to settle adjacent to the dorsal aorta where they constitute the primary sympathetic chain that will mature into the trunk sympathetic chain (Figure 3A; Le Douarin, 1986). Members of the bone morphogenetic protein (BMP) family of proteins derived from the dorsal aorta are thought to be involved in the induction of a number of transcription factors such as the mouse achaete-scute homologue 1 (Mash1) or the paired-like homeodomain transcription factors Phox2a/b (Anderson et al., 1997; Lo et al., 1999; Schneider et al., 1999; Francis and Landis, 1999) that in turn control autonomic neuron specific features (Francis and Landis, 1999; Guillemot et al., 1993; Pattyn et al., 1999; Stanke et al., 1999).

Subpopulations of sympathetic neuroblasts migrate further to generate additional sympathetic ganglia such as the rostrally located superior cervical ganglion (SCG), the ventrally located prevertebral ganglia and chromaffin cells of the adrenal medulla (Figure 3A; Le Douarin, 1986). Both the migration of sympathetic precursors as well as the extension of sympathetic axons towards their target organs occurs in tight association with blood vessels (Figure 3). These findings have led to the suggestion that factors released either by the endothelial blood vessel cells themselves or by the surrounding smooth muscle cells could act as intermediate target-derived cues to direct neuronal migration and sympathetic axon outgrowth. Recent experimental evidence suggests that members of the glial cell line-derived factor (GDNF) and neurotrophin family of ligands and their

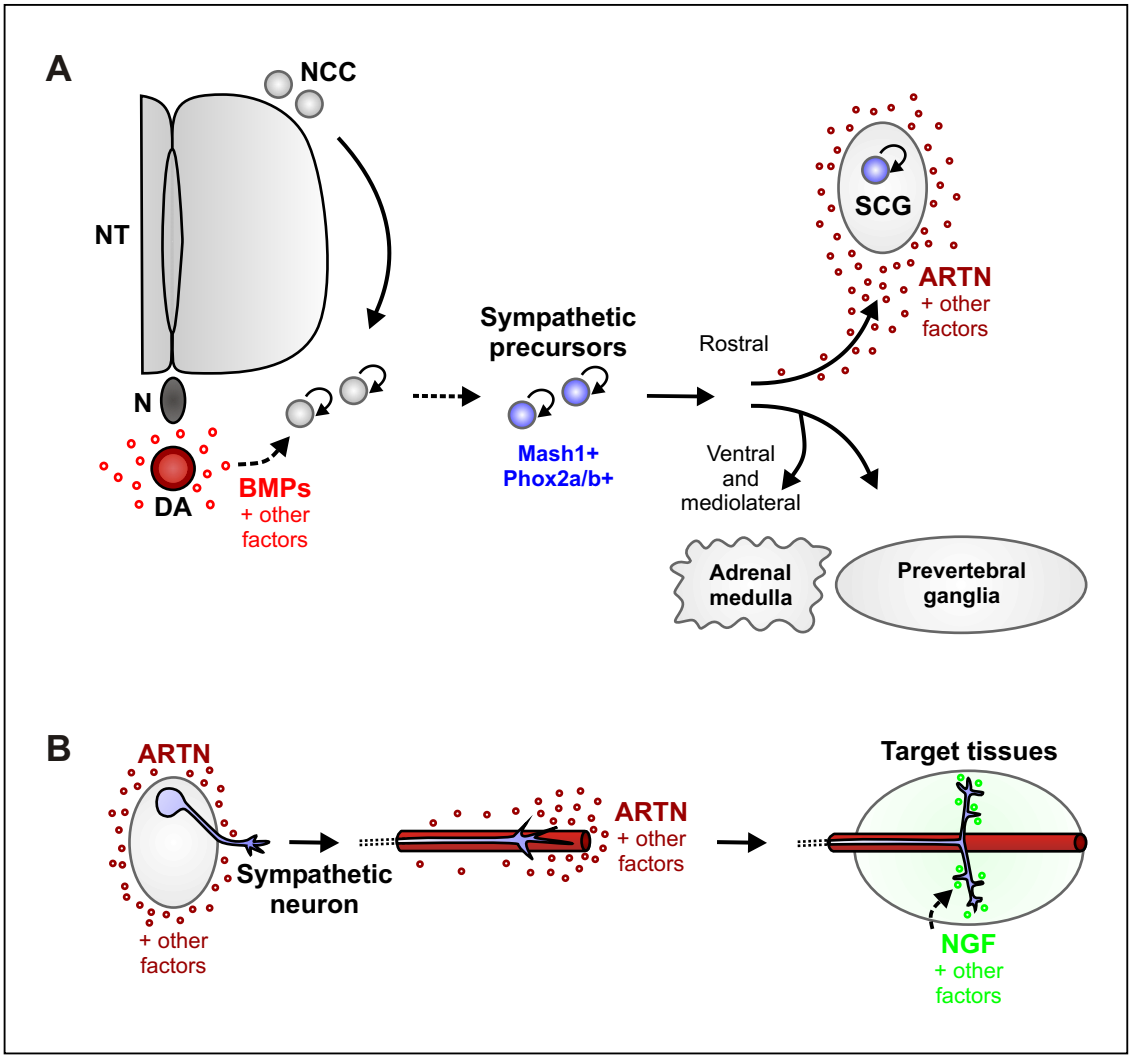


Figure 3. Target-derived Signals and the Differentiation of Sympathetic Neurons. (A) A subset of neural crest cells (NCC), emanating from the dorsal neural tube (NT), migrates ventrally and settles in the vicinity of the dorsal aorta (DA) to form the primary sympathetic chain. Bone morphogenetic proteins (BMPs) secreted by the dorsal aorta lead to the induction of the transcription factors Mash1 and Phox2a/b in sympathogenic precursors. Some of the sympathetic precursors migrate further rostrally in response to artemin (ARTN) to form the superior cervical ganglion (SCG) whereas others migrate ventrally and medio-laterally to give rise to the prevertebral ganglia and chromaffin cells of the adrenal medulla. (B) Intermediate target-derived factors such as ARTN also influence the development of sympathetic axonal projections which form in close proximity to large blood vessels. Once sympathetic axons reach their final targets nerve growth factor (NGF) acts to direct target invasion and terminal axon branching. Abbreviation: N=notochord.

corresponding receptors play important roles in controlling the differentiation of sympathetic neurons (Enomoto et al., 2001; Honma et al., 2002; Glebova and Ginty, 2004).

Artemin (ARTN), a member of the GDNF family ligands binds to the GDNF family receptor α3 (GFRα3), one of four known ligand binding glycosyl-phosphatidyl-inositol (GPI)-anchored GFRα receptors (GFRα1-4; Baloh et al., 2000; Airaksinen and Saarma, 2002). GFRα receptors heteromerize with the common receptor tyrosine kinase Ret upon ligand binding to elicit downstream signaling events (Saarma, 2001). Whereas *GFRα3* is expressed in the entire sympathetic nervous system throughout embryonic development, *ARTN* expression appears to be dynamic (Honma et al., 2002; Nishino et al., 1999). *ARTN* is expressed in the vicinity of the sympathetic chain and within the SCG, around intercostal blood vessels that line the axonal projections of neurons from sympathetic trunk ganglia and in smooth muscle cells of blood vessels of the gastrointestinal tract (Figure 3; Honma et al., 2002; Nishino et al., 1999). Interestingly, *ARTN* expression is initially present in proximal segments of the developing vasculature and only extends to more distal regions as development proceeds, mimicking the time course of sympathetic axon outgrowth. Thus, the spatio-temporal expression patterns of *ARTN* and *GFRα3* are compatible with a role in sympathetic nervous system development.

Analysis of *GFRα3* and *ARTN* mutant mice revealed that this signaling system is indeed involved both in the migration of sympathetic neuroblasts as well as in sympathetic neuron axon outgrowth (Honma et al., 2002). During SCG development, ARTN is first required to direct rostral SCG precursor migration (Figure 3A). Consequently, the absence of ARTN leads to a misplaced SCG. Later, ARTN is essential for SCG axon outgrowth towards the superior tarsus muscle of the upper eyelid (Figure 3B). In addition, the development of other sympathetic axonal projections, such as the outgrowth from prevertebral ganglia or the trunk sympathetic chain is also severely impaired in *GFRα3* or *ARTN* mutant mice, although these phenotypes are partially rescued at later developmental stages (Honma et al., 2002). Similarly, GDNF functions as an intermediate target-derived factor during development of the cranial parasympathetic nervous system (Hashino et al., 2001).

The neurotrophin nerve growth factor (NGF) has also been implicated in the establishment of sympathetic axonal projections, albeit at later developmental stages (Glebova and Ginty, 2004). Since sympathetic as well as cutaneous dorsal root ganglia (DRG) neurons depend on NGF for survival (Francis and Landis, 1999; Farinas, 1999), the investigation of potential survival

independent functions of NGF was based on an elegant genetic strategy to prevent neuronal cell death in these neurons (Glebova and Ginty, 2004; Patel et al., 2000). The analysis of mice mutant for both *NGF* and the pro-apoptotic Bcl-2 family member gene *Bax* revealed that NGF signaling is required in sympathetic neurons for axonal target invasion *in vivo* (Figure 3B). Interestingly, not all sympathetic target tissues exhibit a comparable degree of innervation defects in *NGF/Bax* double mutant mice suggesting that additional target-derived factors expressed in distinct target regions may fulfill analogous roles in target invasion for different subpopulations of sympathetic neurons (Glebova and Ginty, 2004).

Together, these experiments suggest that the primary role for ARTN/Gfrα3 signaling in sympathetic neuron development is to support directed neuronal migration and axon outgrowth rather than to support neuronal survival (Figure 3). However, transcriptional events downstream of ARTN signaling are currently unknown. In contrast, NGF signaling is required in sympathetic neurons both for their survival and at later developmental stages to mediate target invasion (Figure 3B). Whether transcriptional downstream signaling events in sympathetic neurons are also mediated through CREB signaling as has been suggested for NGF dependent cutaneous DRG sensory neurons (Lonze and Ginty, 2002) awaits further investigation.

Peripheral Signals Control the Establishment of the Spinal Monosynaptic Reflex Circuit

Two main neuronal classes are interconnected to form the spinal monosynaptic reflex circuit in vertebrates. Motor neurons in the ventral horn of the spinal cord innervate distinct groups of muscles in the periphery. In turn, they receive monosynaptic input from Ia proprioceptive DRG sensory neurons (Eccles et al., 1957; Brown, 1981). This well studied neuronal circuit represents an easily accessible vertebrate neuronal circuit with limited neuronal complexity and has thus been the focus of many studies (Chen et al., 2003).

It is well established that early steps in the differentiation of motor neurons including initial axon pathfinding decisions are controlled by transcriptional programs independent of peripheral influence (Jessell, 2000; Lee and Pfaff, 2001). It has however also been evident for quite some time that DRG sensory neurons are capable of adjusting their neuronal phenotype and connectivity in response to specific environmental cues. In particular, Ia proprioceptive afferent DRG neurons are thought to establish specific connections with motor neurons projecting to the same muscle through target-derived peripheral signals (Frank and Wenner, 1993; Ritter and Frank, 1999). However, the

molecular components responsible for the specification of central connectivity still remain to be identified, as do the peripheral signals themselves. Recent evidence has now revealed molecular mechanisms through which peripheral signals act on the differentiation of both motor neurons and proprioceptive afferents but at stages prior to synaptogenesis.

Pea3 and Er81 are members of the ETS family of transcription factors (Sharrocks, 2001) and are expressed by distinct subpopulations of motor neurons and DRG sensory neurons (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002). The comparatively late onset of their expression during development coinciding with the time when axons begin to invade their peripheral targets raised the question of whether their induction might be mediated by target-derived cues. Limb ablation experiments in the chick embryo revealed that the initiation of expression of both Pea3 and Er81 in motor neurons and DRG sensory neurons indeed requires the presence of peripheral signals (Lin et al., 1998).

At brachial levels of the spinal cord, motor neurons innervating two distinct target muscles in the periphery (*latissimus dorsi* and *cutaneous maximus*) express Pea3 only upon projection to the periphery (Figure 4A, B). In the absence of Pea3, these motor neurons project to the periphery but fail to innervate their target muscles (Livet et al., 2002). A strikingly similar phenotype is also observed in mice mutant in *GDNF* or in the GDNF ligand binding receptor component *GFRα1* (Haase et al., 2002). Indeed, *GDNF* mutant mice fail to induce Pea3 expression in brachial motor neurons and the peripheral expression of GDNF is spatially coincident with the trajectory of motor neurons innervating *latissimus dorsi* and *cutaneous maximus* muscles (Figure 4A; Haase et al., 2002). Interestingly, this signaling pathway is not only required for the establishment of axonal projections, but also coordinately regulates motor neuron cell body positioning within the spinal cord. In the absence of Pea3, the corresponding motor neuron cell bodies fail to migrate to their appropriate position and do not cluster into motor pools (Livet et al., 2002; Haase et al., 2002). In chick embryos, a functional link between the expression of distinct combinations of Type II cadherins and motor pool clustering has been described recently (Price et al., 2002). The deregulation of at least two cadherins in motor neurons of *Pea3* mutant mice suggests that these may be downstream mediators of Pea3 to control motor neuron cell body positioning (Livet et al., 2002). It is also tempting to speculate that the positioning of motor neuron cell bodies in the spinal cord could influence central connectivity by providing targets for distinct sets of synaptic inputs.

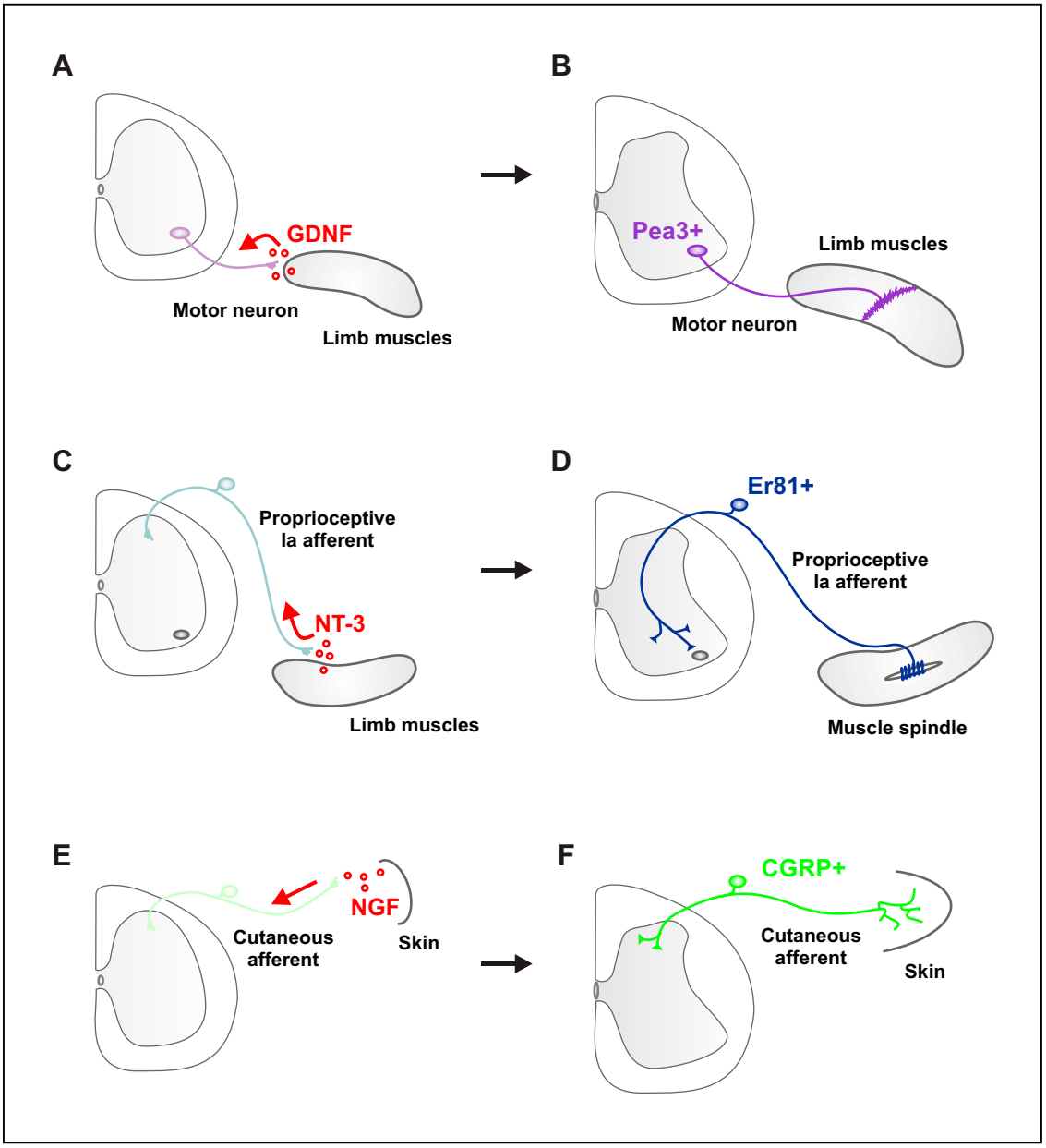


Figure 4. Peripheral Signals Control the Formation of DRG Sensory and Motor Neuron Projections. (A, B) Subpopulations of brachial motor neurons extend their axons towards their target muscles. *En route*, they encounter GDNF (A) which is responsible for the induction of Pea3 in the corresponding cell bodies (B). Pea3 expression in motor neurons is required in turn for target muscle innervation as well as motor pool clustering in the spinal cord (B). (C, D) Proprioceptive Ia afferents extend two axonal branches. The peripheral branch is exposed to target-derived NT-3 (C). NT-3 signaling results in the expression of Er81 in proprioceptive afferents (D) which is necessary for the central Ia afferent branch to invade the ventral horn of the spinal cord. (E, F) Cutaneous afferents project towards their target area in the skin where they are exposed to NGF (E). Retrograde NGF signaling controls the establishment of skin innervation and is involved in the upregulation of the neuropeptide CGRP (F).

The ETS transcription factor Er81 is required in proprioceptive DRG sensory neurons to promote the establishment of axonal projections into the ventral horn of the spinal cord (Figure 4C, D; Arber et al., 2000). In *Er81* mutant mice, Ia proprioceptive afferent projections terminate in the intermediate region of the spinal cord and fail to establish monosynaptic connections with motor neurons. Peripheral neurotrophin NT-3 is capable of inducing Er81 expression in proprioceptive afferent neurons (Figure 4C, D; Patel et al., 2003). When DRG from *Bax* mutant mice isolated at stages before the onset of Er81 expression are cultured without supplemental factors, expression of Er81 is not observed. In contrast, the addition of NT-3 leads to the rapid induction of Er81 in proprioceptive DRG sensory neurons. Consistent with these findings, Er81 protein is not detected in proprioceptive afferents of *Bax/NT-3* double mutant mice in which apoptotic cell death is prevented due to the absence of the pro-apoptotic gene *Bax* (Patel et al., 2003). While central projection defects in these mice are similar to *Er81* mutant mice, peripheral projections are affected more severely than in *Er81* mutant mice. Muscle spindles, the peripheral sensory organs innervated by Ia proprioceptive afferents, cannot be observed in *Bax/NT-3* double mutant mice (Patel et al., 2003) whereas they form initially in *Er81* mutant mice and degenerate only later (Arber et al., 2000). These findings suggest that peripheral NT-3 may also control the induction or activation of transcription factors other than Er81 within proprioceptive afferents. In addition to the role of NT-3 in the induction of Er81 during neuronal circuit assembly, muscle spindle derived NT-3 also has a later function by retrogradely influencing synaptic strength at central synapses between Ia proprioceptive afferents and motor neurons (Mendell et al., 2001; Chen et al., 2003).

Further evidence that neurotrophins play a more general role in the development of sensory projections in a manner independent of their role in regulating neuronal survival came also from the analysis of *Bax/NGF* and *Bax/TrkA* double mutant mice (Figure 4E, F; Patel et al., 2000). These mice show severe defects in the development of peripheral projections and in the innervation of cutaneous target tissues whereas no obvious anatomical defects in the innervation of central targets in the spinal cord were found (Patel et al., 2000).

Target-derived BMPs Control Neuropeptidergic Character in Transcriptionally Pre-specified Neurons in Drosophila

The acquisition of late aspects of neuronal fate by target-derived cues is not restricted to vertebrate species despite the fact that no direct homologues for neurotrophins have been identified in invertebrate species. Beautiful recent work in *Drosophila* has now revealed the existence of signaling pathways through which target-derived signals control maturation of defined subpopulations of neurons (Figure 5; Allan et al., 2003; Marques et al., 2003).

In the *Drosophila* ventral nerve cord, three bilaterally located Tv neuroendocrine neurons innervate three endocrine glands at the midline, the neurohemal organs (NHO). These neurons express a characteristic combination of transcription factors and the neuropeptide FMRFamide (FMRFa; Benveniste et al., 1998; Allan et al., 2003; Marques et al., 2003; Nichols, 2003). Several lines of evidence suggest that the retrograde transport of the target-derived BMP homologue Glass bottom boat (Gbb) is essential for the induction of FMRFa expression in Tv neurons (Figure 5). First, in *tinman* mutants in which the target organ NHO is absent, Tv neurons fail to express FMRFa (Allan et al., 2003; Gorczyca et al., 1994). Second, mutations which prevent Tv neurons from receiving target-derived signals by either misdirecting axonal projections to different targets or by blocking retrograde axonal transport using expression of a dominant-negative form of the dynein-dynactin microtubule motor complex, result in complete absence of FMRFa expression in Tv neurons (Allan et al., 2003; Marques et al., 2003). Third, BMP signaling results in the phosphorylation and nuclear translocation of the Smad homologue Mothers against dpp (Mad). This activation can readily be observed in wild-type Tv neurons as soon as their target NHO is reached but is not found in *tinman* mutants. Compatible with this model, in mutants in the BMP type II receptor *wishful thinking* (*wit*) or the BMP ligand *gbb*, the expression of FMRFa in Tv neurons fails to be induced in Tv neurons despite the fact that their axons reach their target (Allan et al., 2003; Marques et al., 2003). Interestingly, the induction of FMRFa expression in Tv neurons does not only require the presence of Gbb in the target region and the neuronal expression of the receptor Wit but also the concomitant expression of the LIM homeodomain transcription factor Apterous and the zinc finger transcription factor Squeeze within a peptidergic cellular context (Benveniste et al., 1998; Allan et al., 2003). These findings argue for a permissive rather than an instructive role of BMP signaling in FMRFa induction within Tv neurons.

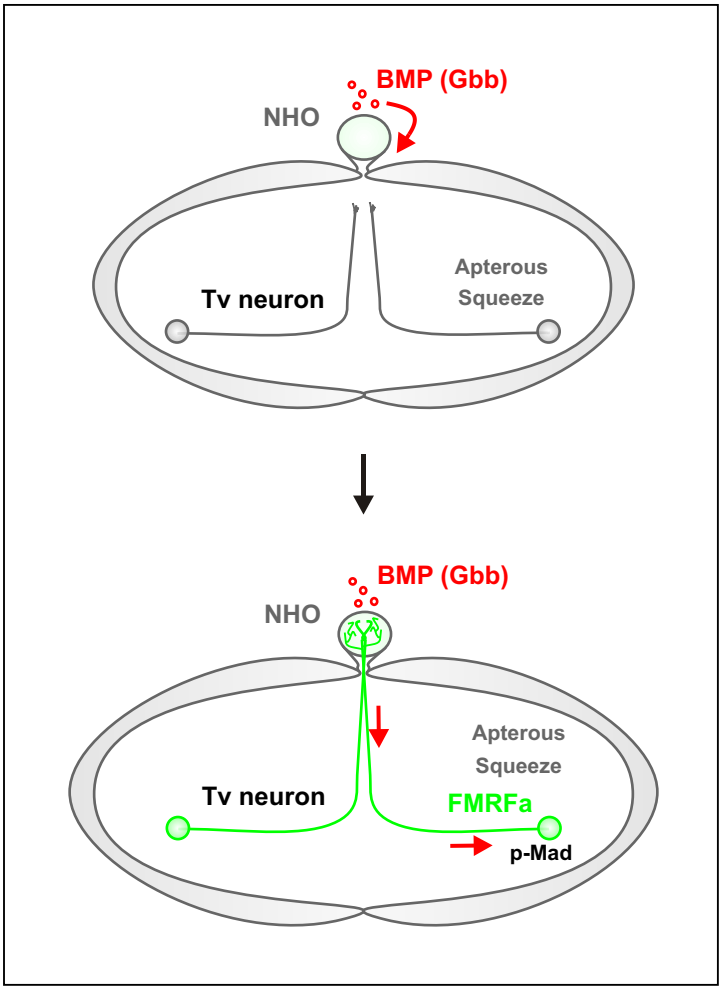


Figure 5. BMP Signaling Controls Peptidergic Neuronal Differentiation in *Drosophila*. In the thoracic segments of the *Drosophila* CNS Tv neuroendocrine cells which express Apterous and Squeeze extend their axons towards the midline and project dorsally to innervate the neurohemal organ (NHO). BMP (Gbb) signals originating from the NHO and overlying mesoderm are transported retrogradely to the Tv neuron cell bodies and are responsible for the phosphorylation and activation of Mad (p-Mad). Transcriptional responses downstream of p-Mad, in combination with the presence of Apterous and Squeeze, result in the upregulation of the neuropeptide FMRFa within Tv neurons.

The regulation of neurotransmitter expression to influence neuronal identity by target-derived signals has also been studied in vertebrates (see for example Nishi, 2003). Sympathetic neurons innervating the rat sweat gland undergo a characteristic developmental switch from noradrenergic to cholinergic and peptidergic neurotransmitter phenotype in response to target-derived signals (Francis and Landis, 1999; Nishi, 2003). While some evidence suggests that members of the cytokine gene family harbor this activity, the endogenous factor mediating this response has not yet been identified (Francis and Landis, 1999; Nishi, 2003). Moreover, also neurotrophins have been shown to regulate the acquisition of neurotransmitter phenotypes, since NGF is required for the induction of CGRP expression in DRG sensory neurons both *in vitro* and *in vivo* (Figure 4F; Patel et al., 2000).

Concluding Remarks: Permissive Signals Instructing Neuronal Circuit Maturation

The final steps in neuronal circuit formation are accompanied by late events in neuronal differentiation such as terminal neuronal cell body positioning, axonal extension and synaptogenesis within the target area as well as the acquisition of mature neuronal properties including the choice of neurotransmitter phenotype. These steps have been shown to depend on interplay between early-acquired neuron-intrinsic transcriptional programs and late target-derived signals as neuronal cell bodies migrate towards their mature positions and axonal growth cones approach their targets (Figure 6).

Interestingly, signaling molecules with a function in target-mediated terminal neuronal differentiation (Figure 7) have often previously been studied for their roles in controlling other biological processes, most notably neuronal survival. Several of these target-derived factors act preferentially on pre-determined subpopulations of neurons which express not only the receptor(s) appropriate for the cognate ligand(s) but also are endowed with cell-intrinsic characteristics rendering them competent to respond to a distinct factor. This principle is evolutionarily conserved: in mouse embryos, GDNF can only induce expression of Pea3 in the subpopulation of motor neurons normally expressing Pea3 but not in all motor neurons expressing GDNF receptor components (Haase et al., 2002). Moreover, DRG sensory neurons do not require GDNF as a peripheral signal to express Pea3 but another, yet to be identified factor (Haase et al., 2002). Similarly, Er81 expression in motor neurons is not regulated by peripheral NT-3 (Patel et al., 2003). In *Drosophila*, the expression of FMRFa in Tv neurons not only requires target-derived

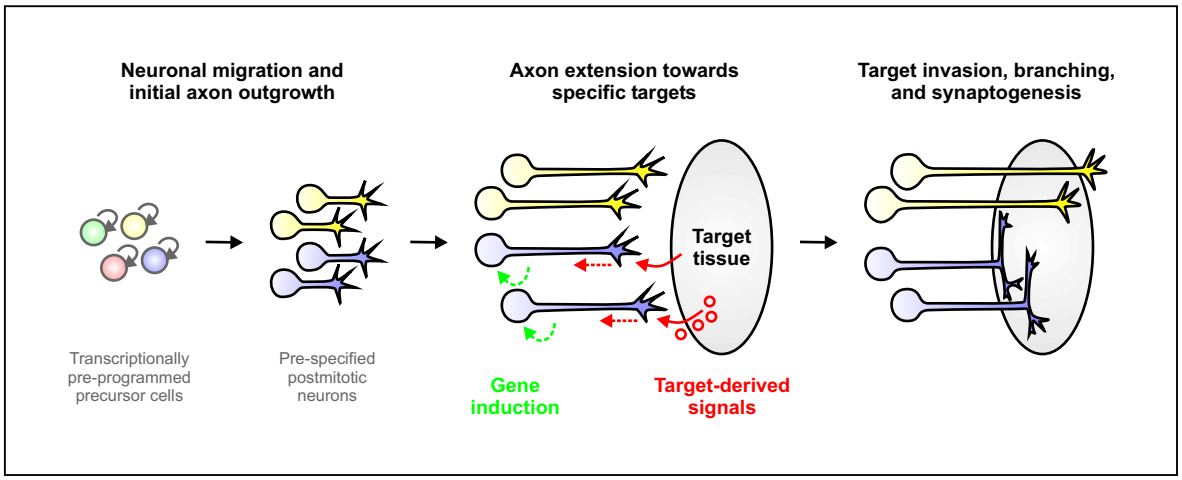


Figure 6. Control of Neuronal Phenotype by Target-derived Factors. Schematic diagram of sequential steps in neuronal differentiation. Neuronal precursors arise from dividing neuroblasts in a spatially and temporally defined manner. These early precursors are transcriptionally pre-programmed enabling them to respond specifically to environmental signals. In the process of axon outgrowth towards their targets, growth cones respond to signals (red) encountered at intermediate targets or final targets. These target-derived signals act permissively on pre-determined neuronal subpopulations (blue) through retrograde signaling mechanisms leading to gene expression changes in the cell bodies, which in turn control target invasion, axonal branching and synaptogenesis. Other neuronal subpopulations (yellow) do not respond to these target-derived cues and continue to grow.

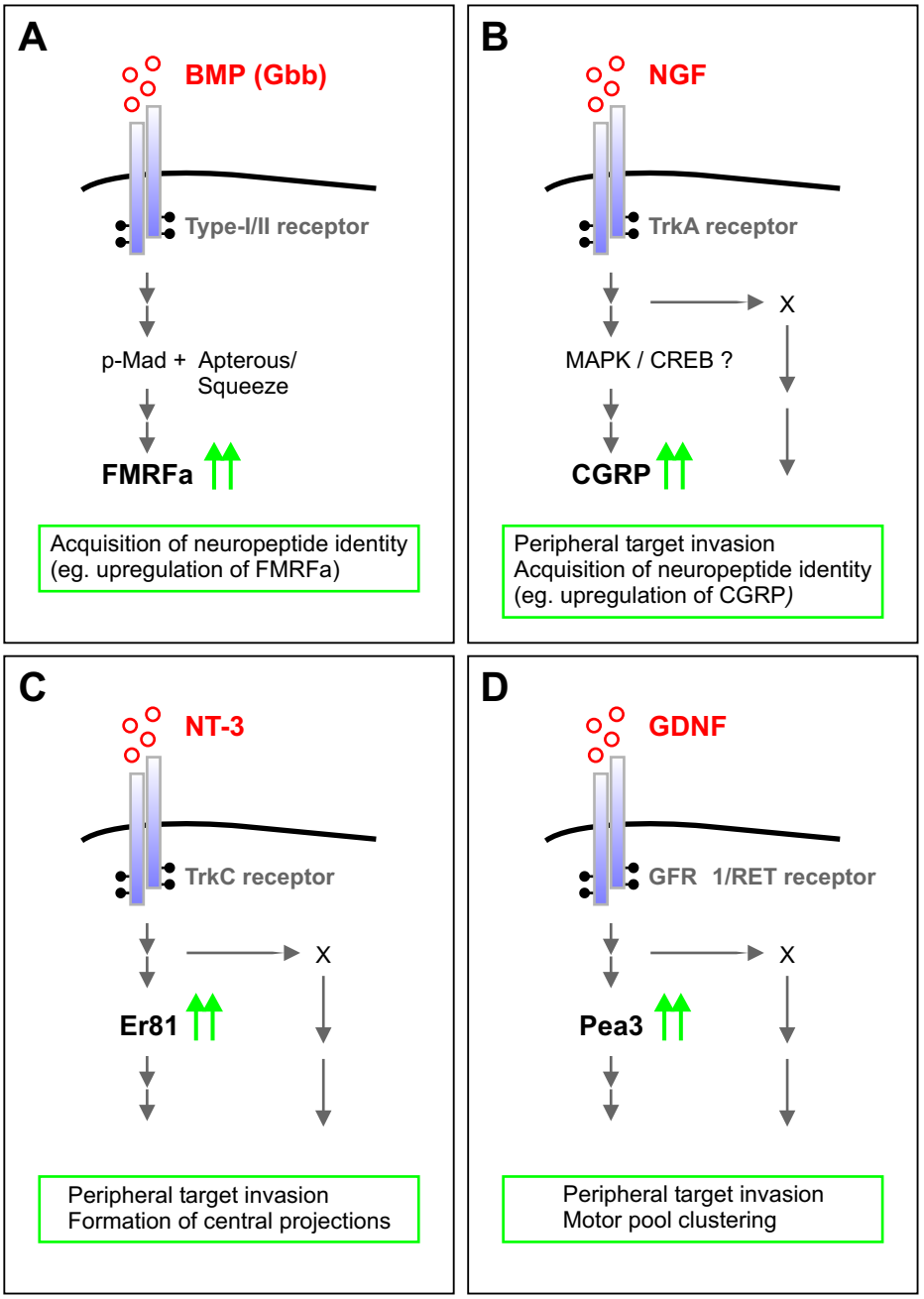


Figure 7. Intracellular Signaling in Response to Target-derived Factors. (A) In *Drosophila*, BMP (Gbb) signals through type-I/II receptor complexes leading to the phosphorylation of the Smad protein Mad (p-Mad) which is involved in the control of transcriptional processes eventually resulting in the upregulation of FMRFa. A prerequisite for the induction of FMRFa is the presence and action of both Apterous and Squeeze. (B) Cutaneous DRG sensory and sympathetic neurons express TrkA which is activated by NGF. MAPK and CREB are downstream targets of the TrkA signaling cascade and could be involved together with additional factors (X) in the upregulation of CGRP and/or aspects of peripheral target invasion of cutaneous and sympathetic neurons. (C) Upregulation of Er81 in proprioceptive afferents requires NT-3 signaling through TrkC. Er81 is responsible for the establishment of central projections into the ventral horn of the spinal cord. Additional factors also regulated by NT-3 (X) might be required for proper peripheral target invasion. (D) GDNF binding to GFR /RET receptor complexes leads to up-regulation of Pea3 in a subset of motor neurons. Pea3 activity (in concert with putative other factors X) is required for peripheral target invasion and motor pool clustering.

BMP, but in addition is dependent on the expression of at least two known transcription factors in a peptidergic neuronal lineage (Allan et al., 2003).

Thus, signaling specificity required for the induction of certain characteristic late neuronal traits appears to be regulated through pre-specification of neuronal subpopulations. The expression of appropriate receptors but importantly also the cell-intrinsic competence to respond to a target-derived signal by activation of a certain downstream program represent key elements to achieve signaling specificity (Figures 6, 7). Upon integration of target-derived signals within neuronal cell bodies, intrinsic genetic programs can be adjusted to the needs encountered by the axonal growth cones as these approach the target area (Figure 6). The recent experiments summarized in this review have only begun to shed light on how this fine-tuned interplay between neurons and targets functions to control neuronal circuit maturation. Exciting work in the future will reveal the full breadth of activities mediated by target-derived factors in neuronal circuit assembly such as synaptic connectivity, ion channel and neurotransmitter receptor expression or elaboration of dendritic morphology.

Chapter 3

Development of the Monosynaptic Stretch Reflex Circuit

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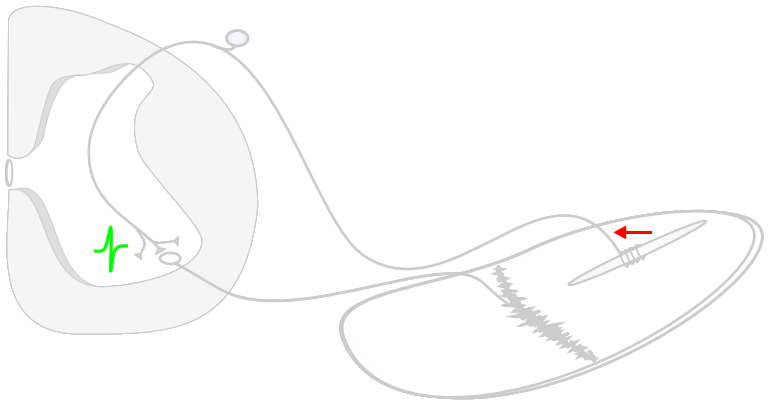


Summary

Significant advances have been made during the past few years in our understanding of how the spinal monosynaptic reflex circuit develops. Transcription factors in the Neurogenin, Runt, ETS, and LIM families control sequential steps of the specification of various subtypes of dorsal root ganglia sensory neurons. The initiation of muscle spindle differentiation requires neuregulin 1, derived from Ia afferent sensory neurons, and signaling through ErbB receptors in intrafusal muscle fibers. Several retrograde signals from the periphery are important for the establishment of late connectivity in the reflex circuit. Finally, neurotrophin 3 released from muscle spindles regulates the strength of sensory-motor connections within the spinal cord postnatally.

Introduction

The monosynaptic stretch reflex circuit is a unique model system for studying the development of a neuronal circuit. Synaptic connections in this circuit are highly precise, yet all of its components are easily accessible for anatomical, functional and genetic investigation. The reflex circuit comprises two distinct functional units: a sensory unit and an effecter unit. The sensory system relays information about the length of a muscle to the CNS. It is composed of relatively few, stretch-sensitive, muscle-embedded mechanoreceptors, known as 'muscle spindles', and specific subpopulations of proprioceptive neurons (Ia afferents) that innervate the muscle spindles peripherally and make excitatory monosynaptic connections to α-motor neurons in the spinal cord. By contrast, the effecter system controls muscle contraction. Its activity is regulated by inputs from the peripheral sensory system, the spinal cord and higher brain centers. It consists of α-motor neurons, each of which projects to a specific muscle and innervates many extrafusal muscle fibers at neuromuscular junctions.

The precise coordination of sensory and effecter systems controls the contraction or relaxation of a given muscle: when a muscle is stretched, the activation of Ia afferents at muscle spindles specifically excites the α-motor neurons projecting to the same or related (homonymous or synergistic) muscles, thereby increasing the tension in the muscle and counteracting the initial stretch. In contrast, motor neurons innervating antagonistic or functionally unrelated muscles receive little or no excitatory input from these Ia afferents. Thus, although the elements of the spinal monosynaptic reflex are interconnected in perhaps one of the simplest and most accessible neuronal circuits known, the selectivity of connections in this circuit makes it an excellent system with which to study the principles underlying the formation of specific synaptic connections.

Recently, significant advances have been made in our understanding of the molecular events that underlie the differentiation of all elements of this circuit. In this review, we focus on those studies and the major steps in this process are shown schematically in Figure 8. References to earlier work can be found in a recent review (Chen and Frank, 1999).

Molecular Specification of Ia Afferents during Development

Ia afferents comprise only a minor fraction of all neurons in the dorsal root ganglion (DRG). A key issue is the nature of the molecular events that control their specification and thus their distinction from other subpopulations of DRG neurons. Although no genes expressed exclusively by Ia afferents have been identified so far, progress has been made in elucidating the early events that control the generation and survival of the proprioceptive neuronal lineage, which includes Ia afferents (Figure 8A).

Proprioceptive afferents express the receptor tyrosine kinase TrkC. Both TrkC and its ligand, neurotrophin 3 (NT3), are required for the survival of proprioceptive sensory neurons (Ernfors et al., 1994; Klein et al., 1994). In contrast, nerve growth factor is required for the survival of TrkA-expressing cutaneous neurons. NT3 is expressed by mesenchyme surrounding the DRG, by motor neurons and by developing embryonic muscles. Much evidence suggests that only muscle derived NT3 is essential for the survival of Ia afferents. The injection of antibodies against NT3 into peripheral tissues causes a decrease in the number of proprioceptive neurons (Oakley et al., 1995), and supplementation of exogenous NT3 rescues the number of Ia afferents in limb-ablated chick embryos (Oakley et al., 1997). In addition, in transgenic mice overexpressing NT3 under the control of a muscle-specific promoter, the number of proprioceptive afferents and muscle spindles increases, and a selective rescue of proprioceptive neurons is achieved in mice lacking endogenous NT3 (Wright et al., 1997; Taylor et al., 2001). These findings suggest that the normal amounts of NT3 made by muscle are insufficient to rescue all of the Ia afferents generated.

Several classes of transcription factors have been implicated in the specification of different classes of sensory neurons, although the extent of our understanding of the transcriptional cascades controlling sensory neuron specification lags behind our understanding of motor neuron specification (Jessell, 2000; Shirasaki and Pfaff, 2002).

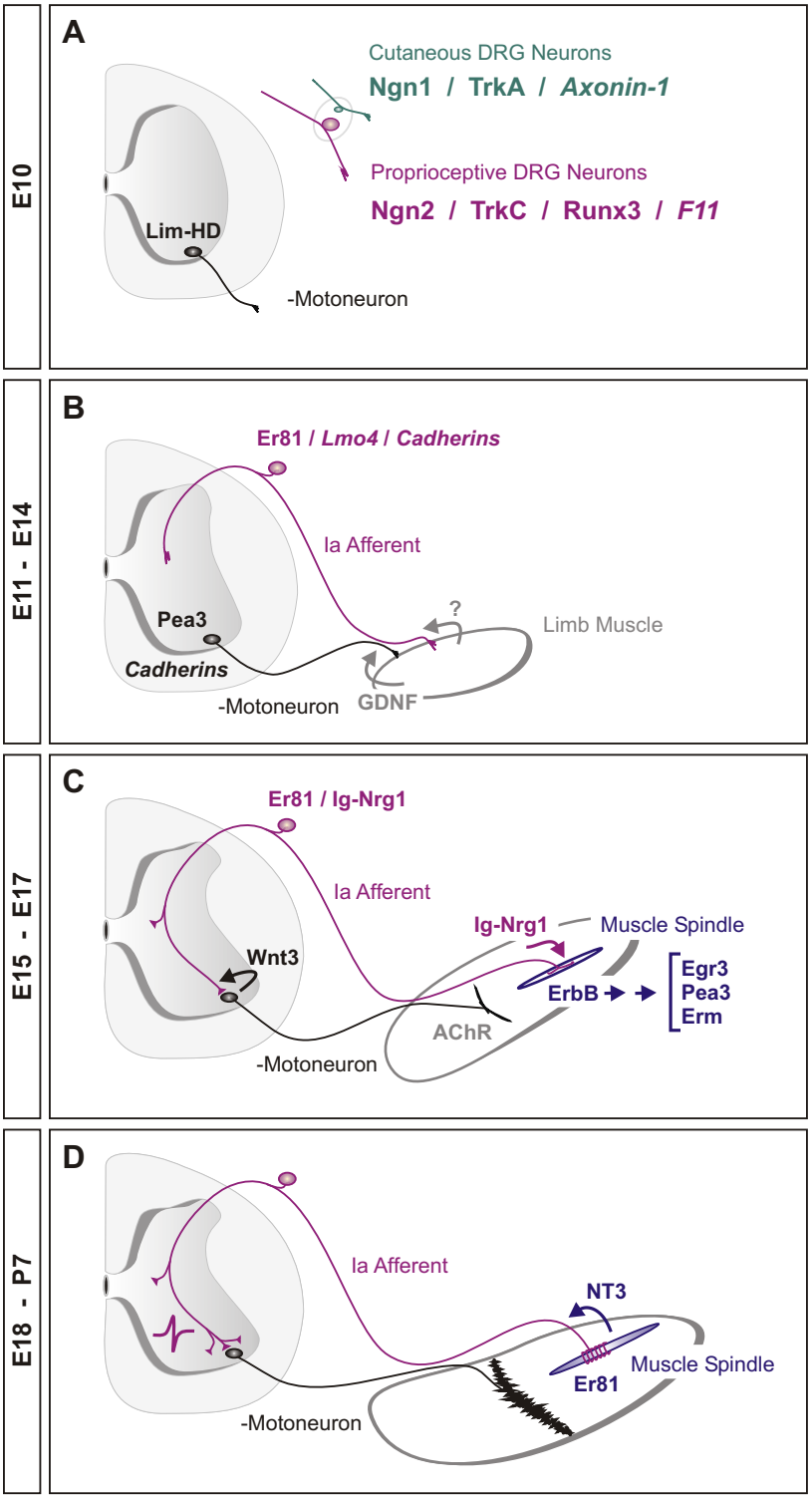


Figure 8. Developmental Assembly of the Spinal Monosynaptic Reflex Circuit. Genes implicated in the progressive steps of this development are shown at four stages, indicated as embryonic day (E) and postnatal day (P) for mice. Proprioceptive DRG neurons are shown in purple, muscle spindles in blue, -motor neurons in black and cutaneous DRG neurons in green. Genes studied mainly in chick embryos are indicated in *italics*. See text for details. Abbreviations: AChR, acetylcholine receptor; GDNF, glial cell line-derived neurotrophic factor; Ig, immunoglobulin; LIM-HD, LIM homeodomain.

First, the combinatorial expression of the basic helix-loop-helix proteins neurogenin 1 (Ngn1) and neurogenin 2 (Ngn2) is essential for the generation of all DRG sensory neurons in mice (Ma et al., 1999). Most proprioceptive neurons are derived from an early Ngn2-dependent precursor population. In contrast, the generation of most cutaneous sensory neurons depends completely on Ngn1. In Ngn2-deficient mice, there is a delay in the generation of proprioceptive neurons (Ma et al., 1999), suggesting that, in the absence of Ngn2, Ngn1-dependent precursors (which are born later) can generate this neuronal population. The issue of how this rescue is achieved has not been addressed as yet.

Second, recent studies suggest that members of the heterodimeric core-binding factor/Runt family of transcription factors are involved in controlling the survival and/or specification of proprioceptive afferents (Levanon et al., 2002; Inoue et al., 2002). These studies show that Runx3 is essential for proprioceptive afferent development and that mice deficient in Runx3 show uncoordinated, ataxic movements, similar to TrkC-, NT3- or Er81-deficient mice (Ernfors et al., 1994; Klein et al., 1994; and see below). Nevertheless, the mechanism of how Runx3 is involved in proprioceptive afferent specification has not been resolved. Depending on the mutant strain, the interpretation points either to a role in the survival of proprioceptive afferent neurons through transcriptional control of TrkC (Ichaso et al., 1998; Levanon et al., 2002) or to a function controlling the development of axonal projections of proprioceptive DRG neurons (Inoue et al., 2002).

Last, the Ets transcription factor Er81 has been implicated in a late step of Ia afferent differentiation. In the absence of Er81, Ia afferents terminate prematurely in the intermediate spinal cord, leading to an almost complete absence of monosynaptic connections between Ia afferents and α-motor neurons (Arber et al., 2000). This study thus provides genetic evidence that a transcription factor induced by peripheral cues controls a late aspect of connectivity of proprioceptive afferents. In future studies, it will be interesting to learn more about the interplay between the different transcription factors and their role in controlling the differentiation of proprioceptive afferent neurons.

Differentiation of Muscle Spindles

(Muscle spindle differentiation is discussed in detail in chapter five)

Many studies have suggested that the differentiation of muscle spindles depends on inductive signals provided by Ia afferents. A series of elegant experiments based on surgical elimination has suggested that sensory, but not motor, innervation is crucial for the maturation of muscle spindles (reviewed in Zelena, 1994). In addition, analyses of TrkC or NT3-deficient mice, in which proprioceptive neurons die before their axons invade the muscle, have revealed a complete absence of muscle spindles, whereas half the complement of muscle spindles is observed in mice heterozygous for NT3 (Ernfors et al., 1994; Klein et al., 1994). The TrkC/NT3 signaling system is not required, however, for the initiation of muscle spindle differentiation. Proprioceptive afferents in the mesencephalic trigeminal nucleus, unlike those in the DRG, do not require NT3 for their survival, and the muscle spindles supplied by these afferents still form normally in NT3- and TrkC-deficient mice (Kucera et al., 1998; Matsuo et al., 2000).

Recent studies have shown that neuregulin 1 (Nrg1) and the ErbB receptor system have an important role in this early inductive interaction between Ia afferents and nascent muscle spindles (Figure 8C; Andrechek et al., 2002; Hippenmeyer et al., 2002). The identification of three transcription factors - the zinc-finger transcription factor Egr3 (Tourtellotte and Milbrandt, 1998) and the Ets transcription factors Pea3 and Erm (Arber et al., 2000), which are expressed selectively by intrafusal muscle fibers even at early developmental stages - has made it possible to monitor early muscle spindle differentiation. The expression of these transcription factors is regulated by Nrg1-ErbB signaling in other biological processes (Burden and Yarden, 1997), making Nrg1 and ErbB receptors good candidates for controlling the initiation of muscle spindle differentiation.

Two main isoforms of Nrg1 can be distinguished: cysteine-rich-domain isoforms are expressed broadly by most or all DRG neurons, whereas immunoglobulin-like isoforms are expressed selectively in the TrkC-expressing proprioceptive afferent population (Hippenmeyer et al., 2002). A receptor for Nrg1, ErbB2, is expressed in intrafusal muscle fibers and the connective tissue surrounding muscle spindles (Andrechek et al., 2002). Conditional genetic elimination of all Nrg1 isoforms from sensory and motor neurons blocks both the initiation of spindle differentiation, including the expression of Egr3, Pea3 and Erm, and the elaboration of Ia afferent terminals at nascent muscle spindles. In contrast, elimination of only the cysteine-rich-domain isoforms does not cause defects in muscle spindle differentiation, suggesting that the immunoglobulin-like isoforms of Nrg1 provided by proprioceptive afferents are sufficient for initiating the differentiation of muscle spindles (Hippenmeyer et al., 2002).

Similarly, deletion of ErbB2 expression in muscle results in severe ataxic behavior and an absence of muscle spindles in the adult, suggesting that this Nrg1 receptor is also required for muscle spindle formation (Andrechek et al., 2002). Despite the fact that Ia afferents do not initiate muscle spindle differentiation in Nrg1-deficient mice, they nevertheless contact individual myotubes (Hippenmeyer et al., 2002). In future work, it will be interesting to determine whether distinct intrafusal precursors are generated that can be selectively recognized by Ia afferents but not by α-motor neurons, an issue that has been debated for many years.

Development of Peripheral and Central Projections of Ia Afferents

To establish a functional stretch reflex, both motor and sensory axons must project to their correct target muscles and the central branches of Ia afferents must terminate in the ventral horn of the spinal cord in the proximity of motor neuron dendrites. Because motor neuron cell bodies supplying a particular muscle are grouped together in the spinal cord, it has been possible using anatomical methods to show that motor neurons are already pre-specified to innervate a specific target region at the time that their axons emerge from the spinal cord (reviewed in Landmesser, 2001).

In contrast, sensory neurons that supply a particular muscle are not clustered together in the DRG, and therefore it has been difficult to demonstrate whether Ia afferents are pre-specified to supply specific muscles and to project into the correct lamina in the spinal cord before they enter their target region. As molecular markers for subsets of proprioceptive sensory neurons have become available (Lin et al., 1998; Chen et al., 2002a; Price et al., 2002), it will be possible to determine whether these genes are expressed before or after sensory axons contact their peripheral targets, and how these genes control the development of peripheral and central projections.

The Ets genes Er81 and Pea3, as well as several members of the cadherin family of genes, fail to be induced after limb ablation (Lin et al., 1998; Price et al., 2002), demonstrating that their expression depends on peripheral signals. Genetic evidence indicates that some of these peripherally regulated genes may be instrumental in controlling late aspects of connectivity (Arber et al., 2000; Livet et al., 2002). Support for this idea is also provided by the localized expression of glial cell line-derived neurotrophic factor, a factor that is required for the induction of Pea3 expression in motor neurons (Haase et al., 2002).

In addition, several surgical experiments suggest that during development the particular target region encountered by Ia afferents not only may be involved in the development of their projections but also may determine the selectivity of their central connections with motor neurons (Wenner and Frank, 1995; reviewed in Frank and Wenner, 1993). To resolve the issue of the role of peripheral targets in controlling reflex connectivity, further studies should be directed towards identifying the peripheral signals that regulate Ia-specific gene expression and the molecular mechanisms that underlie this regulation.

Recent studies have proposed the involvement of two cell-adhesion molecules from the immunoglobulin superfamily in the projection of sensory axons in the spinal cord. In chick embryos, F11/F3/contactin is expressed by proprioceptive DRG neurons, whereas expression of axonin-1/TAG-1 is restricted to nociceptive DRG neurons, whose axons terminate in superficial layers of the spinal cord (Figure 8A; Perrin et al., 2001). The injection of a function-blocking antibody against F11 selectively perturbs the development of Ia projections to the ventral spinal cord. The blockade of NrCAM, which is a binding partner of F11, also disrupts Ia afferent projections, suggesting that interactions between F11 and NrCAM are required for Ia axons to establish their specific projections to the ventral horn.

Once Ia afferents have reached the vicinity of their target region in the ventral spinal cord, they branch extensively as they innervate motor neurons. This branching activity may be mediated, at least in part, by Wnt3 expressed by motor neurons in the lateral motor column (Figure 8C; Krylova et al., 2002). When DRG neurons are cultured in the presence of Wnt3, proprioceptive afferents show significantly larger growth cones and branch more elaborately. In addition, this activity is not present in thoracic spinal cord explants, which is consistent with the restricted expression pattern of Wnt3. Future experiments should address the effects of Wnts on Ia afferent branching in vivo and should identify the factors involved in the branching of Ia afferents at thoracic levels of the spinal cord.

Selective Synaptic Connections Between Ia Afferents and Motor Neurons

Synaptic connections between Ia afferents and motor neurons are highly selective. Afferents supplying a single muscle provide a characteristic pattern of inputs to particular subsets of motor neurons at a given segmental level of the spinal cord. Although synaptic connections in some sensory systems are refined by electrical activity during development, the correct pattern of connections in the spinal reflex circuit is apparent from the earliest times that monosynaptic sensory inputs to motor neurons can be recorded (Frank and Westerfield, 1983; Lee et al., 1988; Mears and Frank, 1997) and is independent of the normal pattern of electrical activity (Mendelson and Frank, 1991). It is therefore likely that these connections are specified by molecular cues that are expressed differentially by Ia afferents and motor neurons that supply different muscles. Recent experiments have identified several gene families whose members are expressed differentially in subsets of sensory and motor neurons, and these genes are therefore potential determinants for synaptic specificity in this system.

Two members of the Ets family of transcription factors, Er81 and Pea3, are expressed selectively in distinct motor pools - that is, in groups of motor neurons supplying individual muscles (Figure 8B, C; Lin et al., 1998; Arber et al., 2000). In chickens, a high degree of coincidence in the expression of Er81 and Pea3 in sensory and motor neurons supplying the same muscle has been described (Lin et al., 1998). Because Ets genes have been proposed to regulate, either directly or indirectly, the expression of homophilic cell-adhesion molecules, this coincidence of Ets gene expression might lead to selective adhesion between homonymous sensory and motor neurons, thereby providing a molecular mechanism for selective synapse formation.

Whereas the expression of Er81 and Pea3 by distinct motor neuron pools is conserved between chicken and mouse, the expression of these factors in DRG sensory neurons seems to be more divergent between the two species (Arber et al., 2000). In mouse, Er81 expression coincides with all proprioceptive afferents and, as a consequence, Er81 deficient mice show connectivity defects in all Ia afferent neurons. The precise identity of the subpopulation of Pea3-expressing DRG neurons is not yet clear, although the expression of Pea3 at late embryonic stages is not as widespread as that of Er81 among the TrkC-expressing afferents. Resolving whether the expression of Er81 and Pea3 in motor neurons contributes to the selectivity of connections between Ia afferents and motor neurons awaits the generation of conditional mutations.

A recent search for genes that are expressed differentially in sensory neurons supplying different muscles in chick embryos has identified a regulator of the LIM family of transcription factors - the LIM-only homeodomain 4 (Lmo4) protein (Figure 8B; Chen et al., 2002a). Although Lmo4 is expressed in most (~85%) TrkC-expressing sensory neurons in the period when sensory-motor connections are forming, Ia afferents that supply a few specific muscles are largely devoid of this protein. Lmo4 is known to inhibit the activity of LIM homeodomain transcription factors by competing with them for binding to the cofactor NLI, which is required for LIM-dependent gene expression. By regulating the transcriptional ability of LIM homeodomain proteins, Lmo4 might provide diversity in the phenotypes of sensory neurons that share common expression patterns of LIM homeodomain factors, including their choice of motor neuronal targets in the spinal cord.

Finally, the actual proteins mediating the selective recognition process between Ia afferents and motor neurons are likely to be cell-surface proteins. Cadherins are prevalent cell-surface homophilic recognition molecules, and analysis of their expression pattern in the brain has led to the proposal that the matching of cadherin expression by presynaptic and postsynaptic neurons may participate in the establishment of regional-specific neuronal connections (Figure 8B; Suzuki et al., 1997). Motor neuron pools of the chick lumbar spinal cord express distinct combinations of type II cadherins, and perturbation of the combinatorial expression pattern by ectopic expression of one of these cadherins, MN-cad, or by dominant-negative approaches has led to the conclusion that a specific pattern of cadherin expression may be required for segregating distinct motor pools during development (Price et al., 2002).

Type II cadherins are also expressed by distinct subpopulations of DRG sensory neurons and for at least two of them, T-Cad and MN-Cad, this expression is correlated with motor neurons supplying the same muscle (Price et al., 2002), suggesting that cadherin expression may contribute to the formation of specific connections between Ia afferents and motor neurons. Recent evidence also suggests that members of the cadherin gene family may be regulated either directly or indirectly by Ets transcription factors (Gory et al., 1998; Price et al., 2002; Livet et al., 2002). Ectopic expression of Er81 in chick spinal cord induces the expression of MN-Cad (Price et al., 2002) and brachial motor neurons in Pea3 mutant mice show deregulation of Cad7 and absence of Cad8 expression (Livet et al., 2002).

Another class of cadherin-related neuronal receptors (CNR) is also expressed in subsets of sensory and motor neurons during development (Carroll et al., 2001). The presence of more than 20 spliced variants of CNR genes and their synaptic localization suggests that CNR might participate in the

selection of specific sensory-motor synapses (Kohmura et al., 1998). Future experiments in which cadherin expression in the sensory-motor system is perturbed should determine how cadherin expression contributes to the formation of specific connections between Ia afferents and motor neurons.

Regulation of Synaptic Strength by Muscle Spindles

Once the correct pattern of synaptic connections between Ia afferents and motor neurons has been established, the strength of these connections must be maintained and perhaps even modulated throughout life. Recent experiments indicate that NT3 produced by intrafusal muscle fibers in spindles is essential for maintaining functional synaptic connections between Ia afferents and motor neurons (Figure 8D). Although muscle spindles are initially generated in Egr3-deficient mice (Tourtellotte and Milbrandt, 1998), they do not produce NT3 (Chen et al., 2002b) and eventually most of them degenerate (Tourtellotte et al., 2001). In these mice, Ia afferents still project into the ventral horn of the spinal cord, but the excitatory postsynaptic potentials (EPSPs) that they evoke in motor neurons are small. If NT3 is provided for several days after birth by intramuscular injection, normal EPSPs are restored (Chen et al., 2002b), showing that NT3 normally provided by intrafusal muscle fibers is required for Ia synaptic function at early postnatal stages.

This requirement for trophic support continues into adulthood. When a muscle nerve is cut in adult cats or rats, depriving sensory neurons of their connection with muscle, the conduction velocity of Ia afferents and their synaptic connections with motor neurons are reduced. Application of NT3 to the central end of the nerve prevents this loss in conduction velocity and synaptic connectivity (Mendell et al., 1999). A possible mechanism for this potentiation is suggested by the observation that the direct application of NT3 to isolated spinal cords of neonatal rats causes a rapid (~20 min) potentiation of the short-latency, AMPA/kainate receptor-mediated sensory input to motor neurons - an action that requires the presence of functional NMDA receptors (Arvanov et al., 2000). But after the first postnatal week, this rapid potentiation can no longer be induced, even though Ia - motor neuronal connectivity still requires NT3 after this time. Restoration of synaptic connections in Egr3-deficient mice by peripheral injections of NT3 also requires several days to develop (Chen et al., 2002b), implying that NT3 is not acting directly on the central connections. These observations suggest that NT3 may regulate synaptic strength by more than one mechanism.

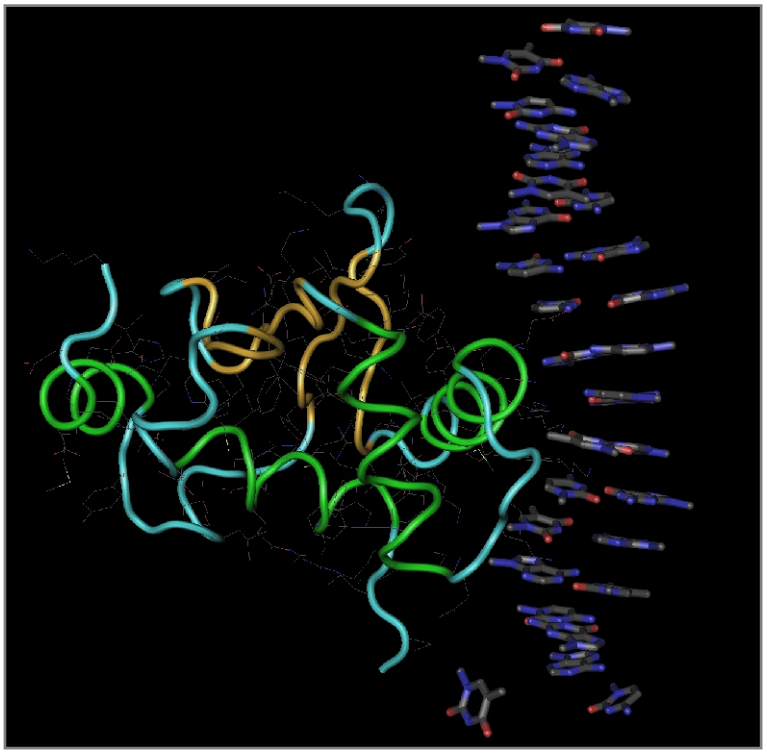
Finally, the strength of synaptic connections between Ia afferents and motor neurons may be actively regulated throughout life. The amount of NT3 released by intrafusal muscle fibers is insufficient to potentiate these synapses maximally, because supplemental intramuscular injections of NT3 during the first postnatal week increase synaptic strength to above normal levels, both in wild-type rats and in Egr3-deficient mice (Seebach et al., 1999; Chen et al., 2002b). Chronic application of NT3 to the cut central ends of muscle nerves in adult cats also potentiates Ia EPSPs above their normal amplitude (Mendell et al., 1999). NT3 release from embryonic muscle has been shown to be dependent on electrical activity in the muscle (Xie et al., 1997). If release of NT3 from intrafusal muscle fibers is similarly dependent on the activity of muscle spindles, this would provide a feedback mechanism for controlling the strength of reflex connections throughout life, depending on the degree of activity of a particular muscle.

Conclusions

Much progress has been made in the past three years in our understanding of the molecular mechanisms underlying the development of the spinal monosynaptic reflex circuit. Distinct subpopulations of motor and sensory neurons seem to be specified by the expression of different families of transcription factors and by the differential expression of cell-adhesion molecules; however, our understanding of these molecular cascades in sensory neurons is only just emerging. Important progress has come also from the identification of neuronally derived signals that trigger the differentiation of muscle spindles, and from the insight that signals from target regions themselves not only are necessary for neuronal survival but also control important aspects of axonal trajectory, target recognition, synaptogenesis and efficacy of synaptic connectivity.

Chapter 4

ETS Transcription Factors
-
Structure & Function



Introduction

In the course of progressive developmental processes, defined subsets genes are activated specifically in a temporally and spatially restricted manner. The process of cellular specification and differentiation thus represents a reflection of changes that are responsible for the induction, maintenance, silencing or suppression of distinct genomic loci. The competence to achieve regulated expression of specific genes is tightly controlled, involving the establishment of dynamic cell-type-specific chromatin patterns (Müller and Leutz, 2001; Kouzarides, 2002), availability and regulated assembly of multiprotein complexes on selected enhancers and promoters (Wolberger, 1999; Merika and Thanos, 2001) and the control of RNA polymerase II mediated transcriptional processes (Maniatis and Reed, 2002). Specificity in the selection of genes to be activated is performed by sequence-specific transcription factors and dependent on the precise arrangement of the DNA-binding sites in the genome but also on the diversity of inter- and intramolecular protein-protein interactions and the signaling cascades impinging on the respective transcriptional regulators (Tjian and Maniatis, 1994; Beckett, 2001; Naar et al., 2001; Taatjes et al., 2004).

The ETS-domain transcription factor family is involved in controlling expression of a variety of genes and plays key regulatory roles in numerous developmental processes including cellular growth, proliferation, differentiation, apoptosis, senescence as well as oncogenic transformation (Macleod et al., 1992; Janknecht and Nordheim, 1993; Sharrocks, 2001; Oikawa and Yamada, 2003)

The first founding member, *v-ets*, of the *ETS* (***Ets***: '***E*** ***t***wenty-***s***ix' or '***E***26 ***t***ransformation-***s***pecific') gene family was initially discovered and identified as a *gag-myb-ets* tripartite fusion gene in the E26 avian erythroblastosis virus, an acutely transforming retrovirus which induces both erythroblastic and myeloblastic leukemias in chicken (Nunn et al., 1983; Leprince et al., 1983). Many cellular homologues were cloned thereafter from a variety of organisms including worms (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*), fish (*Danio rerio*), frogs (*Xenopus laevis*), mice (*Mus musculus*) and humans. *ETS* genes are found exclusively in multi-cellular organisms and probably function in 'evolutionary new' unique regulatory cascades that are specific to the metazoan lineage (Degnan et al, 1993, Laudet et al., 1998, Sharrocks et al., 2001).

The basis and characteristic criteria that defines all ETS family proteins lies within the evolutionarily conserved (ETS) DNA-binding domain (Figure 9A), a region of ~87 amino acid residues that mediates binding to purine-rich DNA sequences (Karim et al., 1990). An invariant

feature of all ETS DNA-binding sites is the occurrence of a central 5'-GGAA/T-3' core consensus motif where sequences flanking this central element contribute to the specificity of binding by distinct ETS proteins (Figure 9B; Nye et al., 1992; Graves and Peterson, 1998). Structural analyses of various ETS proteins revealed that the ETS domain is a variant of the winged helix-turn-helix motif (Liang et al., 1994; Donaldson et al., 1996; Kodandapani et al., 1996) that displays a high degree of structural conservation among different ETS family members (Sharrocks, 2001). In particular, the ETS domain contains three α-helices (α1-α3) and four β-sheets (β1-β4). The main protein-DNA interaction is mediated by the third α-helix that contacts the core GGA tri-nucleotide in the major groove of the DNA (Figure 9A, Kodandapani et al., 1996).

ETS family proteins are divided into several subfamilies (Figure 9C; Laudet et al., 1999; Oikawa and Yamada, 2003; Kurpios et al., 2003) according to their structural composition, sequence conservation within the ETS DNA-binding domain and the presence of additional evolutionarily conserved structural modules or motifs like the Pointed (PNT) domain (Klambt, 1993; Kim et al., 2001). Sequence alignment analyses revealed that different members of distinct subfamilies share a nearly identical ETS domain with in some cases >95% identity on the level of amino acid sequence (Laudet et al., 1999). The human *ETS* gene family includes nearly 30 paralogues to date and orthologues of most of these genes have also been identified in the mouse genome (Kurpios et al., 2003). However, certain *ETS* genes are subject to alternative splicing mechanisms thus generating an array of different isoforms that could inherit additional or different molecular traits (Coutte et al., 1999).

Most ETS transcription factors are downstream nuclear targets of diverse signal transduction cascades including the Ras-mitogen-activated protein kinase (MAPK) signaling pathway (Wasylyk 1998, Yang et al., 2003). Furthermore, additional protein-protein intermolecular interactions, besides upstream signaling input, could result in distinct post-translational modification patterns including phosphorylation (Buchwalter et al., 2004) and probably dephosphorylation (Sugimoto et al., 1997; Tian and Karin, 1999), ubiquitination (Hahn et al., 1997; Chakrabarti et al., 1999), sumoylation (Chakrabarti et al., 2000), and acetylation (Goel and Janknecht, 2003). As a consequence, these diverse interactions and modifications by co-regulatory protein partners result in integrated intramolecular conformational changes of the respective ETS protein that affect DNA-binding activity and/or specificity, transcriptional transactivation or repression properties, association with further transcriptional co-regulators, subcellular localization, and protein stability or degradation.

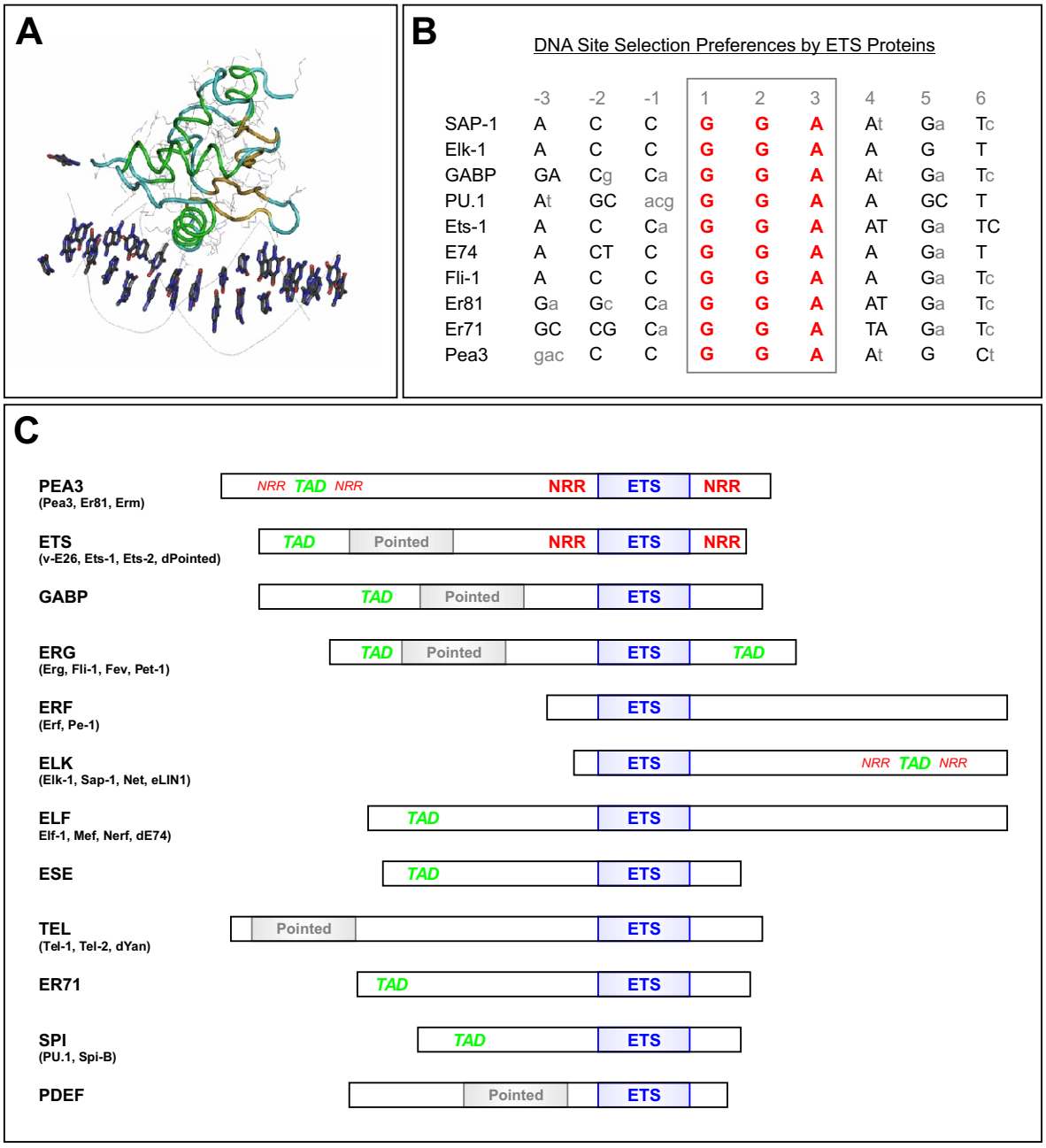


Figure 9. Structural Features of ETS Domain Transcription Factors. (A) Structure of the ETS domain of PU-1 contacting the core GGA triplet in DNA. -helices are in green, -sheets in brown. Note, 3 contacts the major groove of the DNA double helix (from Kodandapani et al., 1996). (B) DNA site selection preferences by ETS proteins. The GGA core sequence is highlighted in red and boxed. Highly preferred bases are indicated in upper case letters (black), and less preferred bases are indicated in lower case letters (grey). Table was adapted from Mo et al., 1998. (C) Schematic diagrams of representative members of each ETS subfamily. The conserved ETS domain is shown in blue, the Pointed domain in grey. Transactivation domains are indicated in green (*TAD*). Negative regulatory regions (red) that affect transactivation (*NRR*) are distinct from negative regulatory regions that repress DNA binding (NRR) and do not share obvious sequence homology or structural similarities. After Laudet et al., 1999; Kurpios et al., 2003, Oikawa and Yamada, 2003.

The vast majority of ETS proteins have been shown to activate the transcriptional machinery. Nevertheless, the ETS family also includes several transcriptional repressors (Mavrothalassitis and Ghysdael, 2000). In addition, particular ETS proteins harbor the intramolecular potential to both activate and repress the transcription of genes where depending on the status of signaling input an ETS transcription factor can switch from being a repressor to an activator (Maira et al., 1996)

ETS Family Proteins and Oncogenic Transformation in Cancer

Oncogenesis results from a multi-step series of genetic changes that lead to alterations in cell physiology such as loss of growth control and normal apoptotic response as well as invasion, metastasis and angiogenesis. Deregulation of *ETS* genes and/or the corresponding protein products has a causative implication in the genesis and progression of various human tumors (Kim and Pelletier, 1999; Shepherd and Hassell, 2001; Kurpios et al., 2003). Several distinct molecular mechanisms account for the deregulation of *ETS* genes in different malignancies. First, Ets proteins might forward signals from oncogenically activated signaling cascades to affect the regulation of proto-oncogenes or tumor-suppressor proteins (Dittmer & Nordheim, 1998). Second, transcriptional up-regulation of expression of *ETS* genes can result in increased levels of the corresponding ETS proteins and therefore ETS specific transcriptional activity (Shepherd and Hassell, 2001; Kurpios et al., 2003). A third molecular pathway that links *ETS* genes to cancer involves specific in frame chromosomal translocations that lead to the expression of active fusion proteins with aberrant but specific properties that are the causal basis for the contribution to malignant transformation of cells (Kim and Pelletier, 1999; de Alava and Gerald, 2000). An enormous array of different cancer types has been linked to various genomic hotspots involved in these chromosomal translocations with *ETS* genes. Two main variants of chimeric ETS fusion proteins as a consequence of chromosomal translocations have been described: First, domains within ETS proteins that represent specific protein-protein interaction interfaces allowing homo- and heterodimerization (eg. Pointed domain) are juxtaposed to intact tyrosine kinase domains from the partner proteins (TrkC, FGFR3, PDGFRβ; Knezevich et al., 1998; Yagasaki et al., 2001; Golub et al., 1994). Second, the ETS DNA-binding domain is fused to a ‘foreign’ transactivation or repression domain of another gene and/or vice versa resulting in the generation of ‘novel’ transcription factors with altered transcriptional properties in comparison to the native parental proteins. The involvement of ETS transcription factors in chromosomal translocations that result in the generation of a hybrid transcription factor with transactivation properties will be further

discussed with particular focus on fusions involving the Ewing's sarcoma proto-oncogene *EWS* and a selection of *ETS* genes.

ETS and Ewing's Sarcoma Tumors

Ewing's sarcoma (ES) and primitive neuroectodermal (PNET) tumors (Ewing's Sarcoma family of tumors, EFTs) affect mostly children or young adults in the first two decades of life and are characterized by small round cells that are sometimes focally arranged as rosettes (Arvand and Denny, 2001, Burchill, 2003). These tumors are poorly differentiated, small, often found associated with bones but pose difficult diagnostic problems when examined by light microscopy. Despite extensive studies, the exact cellular origin of EFTs remains unknown. However, the current opinion in the cancer field is that Ewing's family tumors derive from neural crest progenitors (Arvand and Denny, 2001). This hypothesis is based mainly on the observation that Ewing's sarcoma cells sometimes exhibit neuronal differentiation and that several neural associated proteins are expressed in different EFT derived cell lines (Arvand and Denny, 2001). Furthermore, the finding of peripheral neural crest progenitors late in development differentiating along neural, glial, and mesenchymal lineages might suggest a possible cellular source for EFTs (Cavazzana et al., 1987; Morrison et al., 1999; Arvand and Denny, 2001). However, although the diagnosis of EFTs is often problematic on a histological basis there is a striking cytogenetic characteristic associated with and common to all Ewing's sarcoma tumors, a specific recurrent reciprocal chromosomal translocations involving the Ewing's sarcoma proto-oncogene *EWS* located on the chromosome 22 (Kim and Pelletier, 1999; Burchill, 2003)

The EWS protein shares unique molecular properties and extensive sequence similarity with the related proteins TLS/FUS, $hTAF_{II}68$ in humans (Bertolotti et al., 1996), and SARFH/Cabezza in *Drosophila* (Immanuel et al., 1995; Stolow and Haynes, 1995). On the basis of homology of the primary amino acid sequence, the EWS coding region can be divided into different domains (Figure 10A). The first seven exons encode the N-terminal domain (NTD), rich in glutamine and serine-tyrosine dipeptide motifs. The EWS and $hTAF_{II}68$ proteins have been found to be complexed with both the TFIID and RNA polymerase II fractions in nuclear lysates via their N-terminal domains (Bertolotti et al., 1996, 1998; Petermann et al., 1998). Furthermore, it has been shown recently that the NTD of EWS is indeed capable to stimulate and directly transactivate the transcriptional machinery (Rossow and Janknecht, 2001). The C-terminal exons of *EWS* encode various RNA and single-stranded nucleic acid-binding motifs (Figure 10A). A role for EWS like

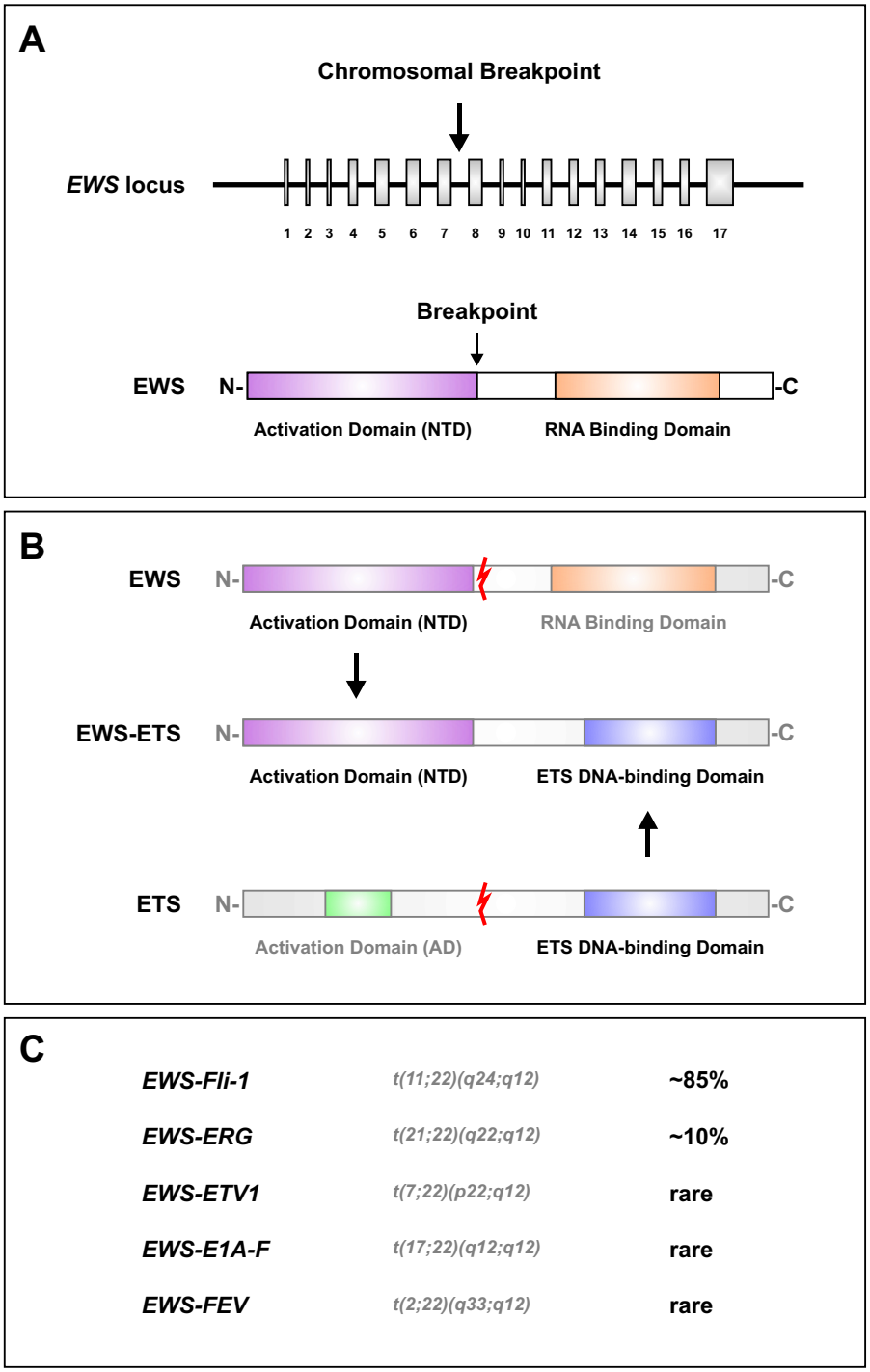


Figure 10. Chromosomal Translocations Lead to Oncogenic EWS-ETS Chimeric Fusion Proteins. (A) Schematic representation of genomic organization of the *EWS* gene and structural features of the EWS protooncoprotein. (Top) The *EWS* genomic locus is located on chromosome 22 and contains 17 exons. ~90% of *EWS* chromosomal breakpoints occur within intron 7 (indicated by black arrow). (Bottom) The N-terminal transactivation domain (NTD) is colored in purple and the C-terminal RNA binding domain in orange. (B) Schematic diagram of EWS-ETS chimeras. In all clinical cases reported, the NTD of EWS is fused to the ETS domain of one of a small selection of ETS transcription factors. (C) Reported *EWS-ETS* fusions as a result of chromosomal translocations (indicated in grey) involving *EWS* and either of the *ETS* genes *Fli-1*, *ERG*, *ETV1*, *E1A-F*, or *FEV*. The frequency (%) of clinical cases is indicated (de Launoit et al., 1997; Kim and Pelletier, 1999).

proteins in splicing has been proposed according to the recent finding that the C-terminus of EWS and TLS/FUS, $hTAF_{II}68$ proteins interact with different splicing factors (Yang et al., 2000). In summary, EWS and the related TLS/FUS, $hTAF_{II}68$ proteins are proposed to act as adapters between transcription and mRNA processing by interacting with components of the transcription apparatus and splicing factors. However, the physiologic significance of these wide ranging associations found in isolated experimental *in vitro* setups will still have to be established.

In the highly malignant Ewing's sarcoma tumors the 5' end (NTD, exons I-VII) of the *EWS* gene is fused to the 3' located ETS DNA binding domain of the *ETS* genes *Fli-1* (~85% of clinical cases), *ERG* (~10%), *FEV* (~1%), or the Pea3 subfamily members *ETV1* (*Er81*, ~1%) and *E1A-F* (*Pea3*, ~1%) (Figure 10B, C; Kim and Pelletier, 1999). The predominant fusion *EWS-Fli-1* has been studied most extensively and some molecular properties of this chimeric protein have been described. In contrast to the native Fli-1 protein, the EWS-Fli-1 fusion is a stronger transcriptional activator (May et al., 1993). In addition, the EWS-ERG, and EWS-ETV1 have also been shown to harbor an increased transactivation potential when compared to ERG and ETV1, respectively (Ohno et al., 1994; Fuchs et al., 2003). Deletion of the EWS transcriptional transactivation domain reduces the biological potency of EWS-ETS proteins (Lessnick et al., 1995). However, other strong transcriptional activation domains can functionally replace the N-terminus of EWS in model systems. Expression of chimeric constructs containing the herpes virus VP16 transcriptional activation domain fused to the Fli-1 C-terminus renders NIH3T3 cells anchorage independent (Arvand and Denny, 2001).

The EWS-Fli-1 chimeric protein displays the same DNA-binding specificity and affinity as Fli-1 (Mao et al., 1994). Furthermore, the EWS-ETV1 fusion protein has been shown to have sequence specific DNA-binding activity depending on the full ETV1 ETS domain (Jeon et al., 1995). Conservation of the ETS domain in the above mentioned translocations coupled with the finding that this domain is required for transformation by EWS-ETS, suggests that sequence specific DNA-binding is maintained and functionally important in all described EWS-ETS fusion proteins (May et al., 1993; Bailly et al., 1994; Jeon et al., 1995). Furthermore, it has been anticipated that expression of distinct structurally divergent fusions in a phenotypically similar tumor might share a subset of target genes responsible for the induction of oncogenic signaling pathways (Jeon et al., 1995; Arvand and Denny, 2001). Despite few genes have been shown to be similarly up- or down regulated by EWS-Fli-1, EWS-ERG, EWS-ETV1 and EWS-E1A-F (Arvand and Denny, 2001; Oikawa and Yamada, 2003), a real direct target gene with physiological significance, contributing actively to the malignant phenotype, remains to be identified.

Together, these findings indicate that the fusion of the NTD of EWS to the ETS domain of a distinct ETS transcription factor results in an aberrant hyperactive ETS DNA-binding sequence specific transcription factor.

The Pea3 Subfamily of ETS Transcription Factors

The Pea3 group is composed of three members: Pea3 (Polyoma enhancer activator 3 or Ets translocation variant 4, ETV4 or adenovirus E1A enhancer binding protein, E1A-F; Xin et al., 1992), Er81 (Ets translocation variant 1, ETV1; Brown and McKnight, 1992; Jeon et al., 1995; Monte et al., 1995) and ERM (Ets translocation variant 5, ETV5 or Ets related molecule Pea3-like; Monte et al., 1994). Each Pea3 subfamily gene is located on a different chromosome (Jeon and Shapiro, 1998) but the three genes display a common architecture comprising 14 exons that encode very similar sequences of the respective protein (Jeon and Shapiro, 1998; Coutte et al., 1999). The three genes encode proteins of about 500 amino acids sharing an overall sequence identity of ~50%. More specifically, these three proteins are more than 95% identical in the ETS domain, more than 85% identical in the 32-residue acidic domain (AD) located in the amino-terminal part, and almost 50% identical in the final 61 amino acids corresponding to the C-terminal tail (CT). Furthermore, several additional functionally conserved regulatory elements, including cis-acting autoinhibitory modules, within Pea3 subfamily proteins have been described but these differ significantly with respect to sequence homology (de Launoit et al., 1997).

Functional analyses have revealed that all Pea3 subfamily members bind to very similar DNA-binding site sequences (Figure 9B; Mo et al., 1998) and commonly activate gene transcription. The conserved AD and CT most likely act synergistically but are both also independently responsible for the transactivation properties of Pea3 (de Launoit et al., 1997), Er81 (Janknecht, 1996) and Erm (Laget et al., 1996). The N-terminal acidic domain, however, is functionally related to acidic transactivation domains such as that of the VP16 viral protein (Laget et al., 1996). Using biophysical circular dichroism methods it has been shown that the 15 initial residues of the AD are fold into an α-helix, which is crucial for transactivation capacity (Defossez et al., 1997). This α-helix is genetically encoded by a single exon and evolutionarily conserved in Erm, Er81 and Pea3 suggesting that this intramolecular feature is essential for all members of the Pea3 subfamily to transactivate gene transcription.

The mechanism of transcriptional activation might include common and/or specifc intermolecular interactions of the conserved AD with components of the basal transcriptional machinery such as TFIID, comprising TBP and TAFs. Erm has been described to interact with such pre-initiation complex components like TAFII60, TBP and TAFII40. Interestingly, TBP and TAFII40 are bound by domains different from the AD in Erm, suggesting that subtle differences in recruiting and targeting distinct general transcriptional activators of the basal transcription machinery, might account for transactivational specificity among Erm, Er81 and Pea3 (Defossez et al., 1997).

Specificity of Action of Pea3 Subfamily ETS Domain Proteins

The existence of a large family of regulatory ETS transcription factors that share highly conserved DNA-binding domains and overlapping DNA-binding sites leads to a specificity paradox in transcriptional regulation (Verger and Duterque-Coquillaud, 2002). However, the specificity problem might be circumvented through several levels of regulation including spatial and temporal specific expression, target-site selectivity, combinatorial control and most important, tight regulation of functional activity (Verger and Duterque-Coquillaud, 2002).
Alternatively, different members of a particular transcription factor (sub-) family could play redundant roles in gene regulation. However, genomic disruption approaches with respect to distinct subfamily members often reveals both redundant and individually exclusive functions for the respective transcriptional regulators (Sharrocks, 2001; Kurpios et al., 2003). This specificity versus redundancy issue is of fundamental importance to the Pea3 subfamily of ETS transcription factors as they are highly homologous with respect to amino acid sequence and sometimes even co-expressed within defined cell populations *in vivo* (Baert et al., 1997; Lin et al., 1998).

Signaling Input to Pea3 ETS Domain Proteins

Intermolecular interactions with co-regulatory protein partners and distinct input from signaling cascades have recently been described to contribute to the specificity of action of individual Pea3 subfamily members and will be discussed with respect to Pea3, Er81 and Erm. These interactive regulatory pathways and protein networks often control the activation or potentiate the transcriptional transactivation potential of Pea3 subfamily proteins through posttranslational modifications (Sharrocks, 2001).

The transcription capacities and status of activation of the Pea3 subfamily members Pea3, Er81 and Erm are regulated and in most cases increased in response to phosphorylation via RTK (receptor tyrosine kinase) signaling input leading to the activation of components of the growth factor and stress activated MAP kinase cascades, Ras, Raf-1, MEK and the MAPK ERK-1 and ERK-2, as well as JNK and p38-MAP kinase (Janknecht, 1996; O'Hagan et al., 1996; Brown et al., 1998). However, not all phosphorylation sites in Pea3, Er81 and Erm are directly targeted by MAPKs. Rather, MAPK-ERK downstream kinases such as the 90-kDa ribosomal S6 kinase 1 (RSK1) has been shown to directly promote phosphorylation of Er81 (Wu and Janknecht, 2002). Further, MK2 (mitogen-activated protein kinase-activated protein kinase 2, MAPKAP kinase 2) that is stimulated via the p-38-MAP kinase pathway directly phosphorylates specific serine residues within Er81 but interestingly results in suppression rather than activation of Er81 (Janknecht, 2001).

In addition to activation by MAPKs, Pea3, Er81 and Erm are targeted and phosphorylated by protein kinase A (PKA) although there appears to occur some species linked specificity among the different Pea3 subfamily members. Especially for Pea3 it has been demonstrated that not every PKA phosphorylation site is conserved across the phylogenetic tree (Baert et al., 2002). Nevertheless, PKA is activated frequently by neurotransmitters or hormones acting via G-coupled receptors and therefore potentially widens the signaling input (besides RTKs) on Pea3 ETS proteins.

A general and frequently observed mechanism contributing to the specificity of various ETS proteins is intrinsic autoinhibition to prevent promiscuous DNA binding and/or inappropriate action of the respective proteins in the absence of specific signaling triggers (for review see Pufall and Graves, 2002). Pea3 contains cis-regulatory DNA binding inhibitory motifs on either side of its ETS domain that prevent DNA binding to a large extent in the absence of appropriate regulatory proteins and/or proposed potential posttranslational modifications (Brown et al., 1998; Bojovic and Hassell, 2001; Greenall et al., 2001). The basic helix-loop-helix-leucine zipper (bHLHZip) transcription factor USF-1 has recently been identified to relieve the autoinhibition of Pea3 and promote DNA binding in a cooperative manner (Greenall et al., 2001). Conversely, members of the Id subfamily of helix-loop-helix (HLH) negatively act in trans on Pea3 to reduce DNA binding (Greenall et al., 2001). Despite autoinhibition of Erm and Er81 has been postulated on the basis sequence conservation, regulators that specifically control DNA binding remain to be identified.

Some proteins that potentiate and/or strengthen the intrinsic transactivation ability of Pea3 have also been described. The AP1 protein c-Jun, β-catenin, and LEF-1 synergize actively with Pea3 to transactivate a luciferase reporter driven by the matrilysin (Matrix metalloproteinase (MMP) 7) promoter. Despite the fact distantly related Ets proteins were not capable of substituting Pea3 the closely related Er81 and Erm both increased luciferase expression and rendered the promoter responsive to β-catenin-LEF-1 and c-jun. These and further observations led to the suggestion that the Pea3 subfamily might act in conjunction with β-catenin-LEF-1 to upregulate the transcription of the matrilysin gene during intestinal tumorigenesis (Crawford et al., 2001).

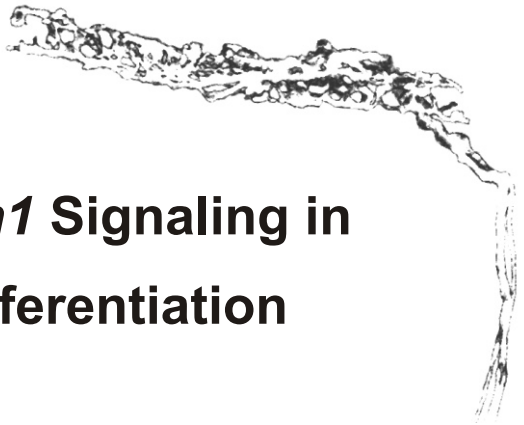
Recent findings suggest that Er81 regulates gene transcription through interaction and association with the homologous global transcriptional coactivators CREB-binding protein (CBP) and p300 (CBP/p300; Papoutsopoulou and Janknecht, 2000). In addition, Er81 is acetylated by the two coactivators/acetyltransferases p300 and p300- and CBP-associated factor (P/CAF) at two specific lysine residues located in the transactivation domain (Goel and Janknecht, 2003). Furthermore, there is evidence that both acetylation and phosphorylation are jointly required and contribute to achieve maximal transactivation of Er81 (Goel and Janknecht, 2003). Two members of the p160 steroid receptor coactivator family, activator ACTR (activator of tyroid and retinoic acid receptor), GRIP-1 (glucocorticoid receptor-interacting protein-1) also acetylate Er81 but mainly augment Er81 mediated transcription in collaboration with p300 (Goel and Janknecht, 2004). Most strikingly, the action of ACTR also led to increased activity of Pea3 and Erm and was very specific only for Pea3 subfamily but no other ETS domain protein (Goel and Janknecht, 2004).

In summary, different signaling cascades lead to a variety of posttranslational modifications including specific phosphorylation and acetylation events in Pea3, Erm and Er81. Together with co-regulatory proteins binding to the Pea3 proteins, these intermolecular and intramolecular interactions fulfill crucial roles in the regulation of specific DNA-binding and transcriptional transactivation properties of the Pea3 subfamily of ETS transcription factors.

Conclusion

While the functional molecular features and properties of the Pea3 subfamily of ETS transcription factors are being resolved in great molecular detail in isolated *in vitro* and cell culture assays there is a substantial need to address issues concerning the functional molecular specificity of these proteins in the regulation of distinct biological processes *in vivo*. The physiological *in vivo* significance and relevance of the numerous intra- and intermolecular interactions involving members of the Pea3 subfamily of ETS transcription factors mostly awaits the proof of concept. However, expression analyses indicate that several of the interacting partners are actually coexpressed with Pea3 ETS proteins in common cellular structures. In particular, all three Pea3, Er81, and Erm are expressed within neural crest derived structures during development (Lin et al., 1998; Arber et al., 2000; Paratore et al., 2002). Interestingly, ID family proteins (Martinsen and Bronner-Fraser, 1998), CBP/p300 (Partanen et al., 1999), and β-catenin-LEF-1 (Bronner-Fraser, 2004) all have been shown to either be expressed or play a role in differentiation or development of various neural crest derivatives although the expression profile at resolution of the single cell level is missing in some cases.

Chapter 5



A Role for *Neuregulin1* Signaling in Muscle Spindle Differentiation

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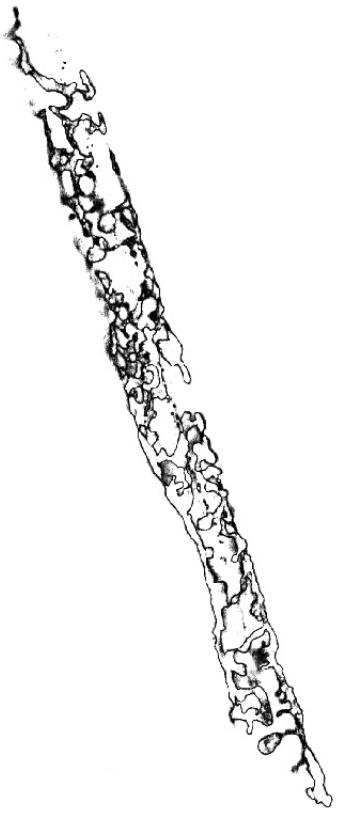

SUMMARY

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 have 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. Amongst 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 which 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 *Er81*, 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*, *Er81* 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., 2000; 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., 2000; 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 post-synaptic 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 et al., 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 different murine *Nrg1* and *ErbB2* mutations. 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 addition, ablation of the ErbB2 signaling subunit of ErbB receptor complexes from skeletal muscle fibers results in similar defects with respect to muscle spindle differentiation as when Nrg1 expression is absent from proprioceptive DRG neurons. In contrast, muscle spindle differentiation proceeds normally in mice that selectively lack *CRD-Nrg1* isoforms. These assays reveal a critical role for Nrg1/ErbB2 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 (Figure 11A-I; data not shown). From E15.5 to at least E18.5, expression of *Erm*, but not of *Pea3* or *Egr3* was also detected in extrafusal muscle fibers (Figure 11C, F, I, J), in a domain that appeared to coincide with the synaptic endplate band, as assessed by the localization of GAP-43^{+} axonal terminals and by the position of high-density clusters of acetylcholine receptors labelled by α-bungarotoxin (BTX) binding (Figure 11K, L). 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 (Figure 12; 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.

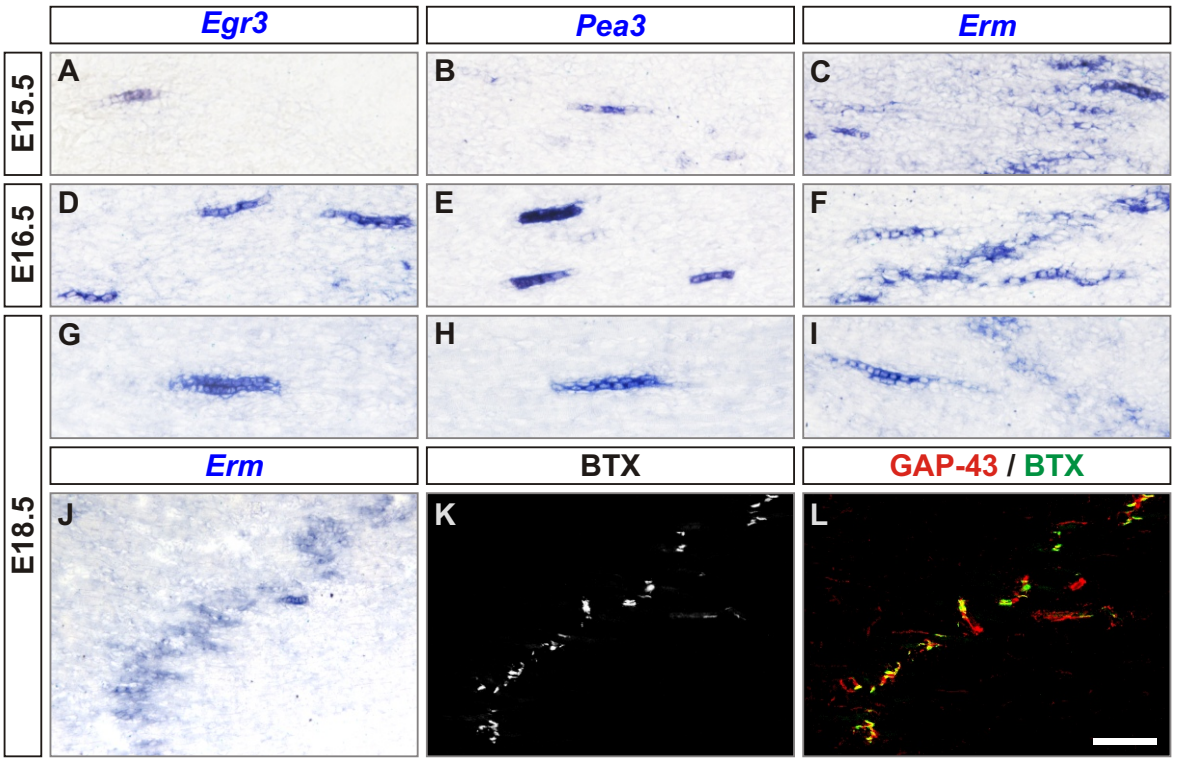

Figure 11. Transcription Factor Expression by Embryonic Muscle Spindles. (A-I) Time course of *Egr3* (A, D, G), *Pea3* (B, E, H) and *Erm* (C, F, I) expression by intrafusal muscle fibers of hindlimb muscles of wild-type embryos at E15.5 (A-C), E16.5 (D-F), and E18.5 (G-I). (J) Expression of *Erm* by myonuclei located within the synaptic endplate band at E18.5. (K, L) Double label immunocytochemistry on adjacent section to (J) to reveal clusters of AChRs with -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, I) in addition to its expression in intrafusal muscle fibers. Scale bar: (A-I) = 40 m; (J-L) = 80 m.

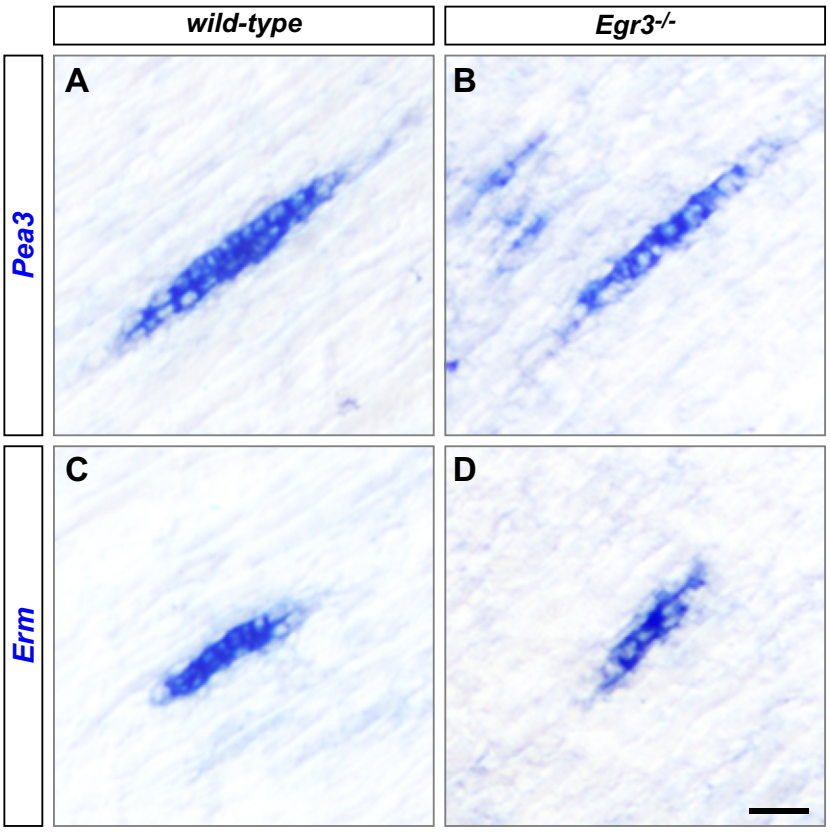

Figure 12. Gene Expression in *Egr3*$^{-/-}$ Mutant Mice. (A-D) Expression of *Pea3* (A, B) and *Erm* (C, D) by intrafusal muscle fibers of hindlimb muscles of wild-type (A, C) and *Egr3*$^{-/-}$ mutant (B, D) embryos at E17.5. Scale bar = 15 m

Proprioceptive Innervation of Nascent Muscle Spindles

Proprioceptive afferents have been implicated in the induction of muscle spindle differentiation (Zelena, 1994; 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 (Figure 13A-C). At this stage, PV^+ axons had made initial contacts with myofibers, and had elaborated rudimentary terminals in a region marked by $Egr3^+$ nuclei (Figure 13A-C). From E15.5 to E16.5, PV^+ axons branched at the central domain of nascent intrafusal muscle fibers (Figure 13D, E; Arber et al., 2000). From E16.5 to P3, proprioceptive axons developed more elaborate annulospiral endings around the central domain of intrafusal muscle fibers (Figure 13F, G). 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 (Figure 13H, I, K). 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 (Farinas, 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.

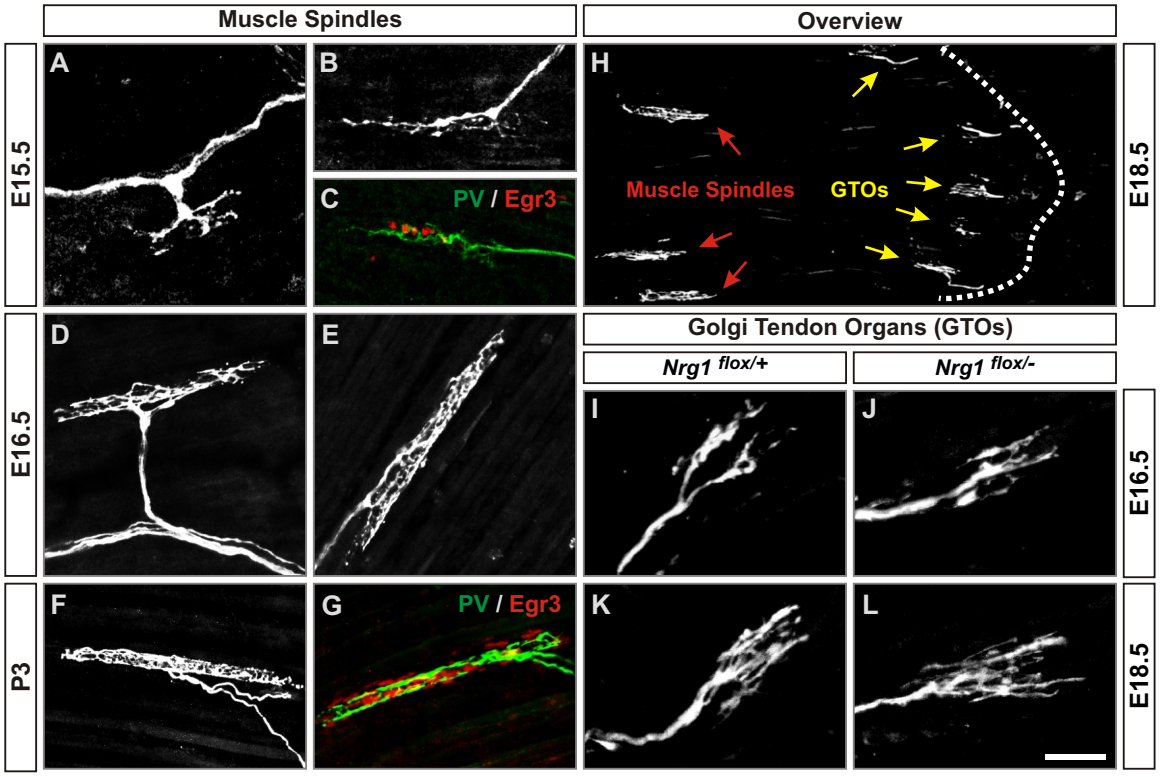

Figure 13. Proprioceptive Afferent Morphology in Developing Hindlimb Muscles. Proprioceptive afferent terminal elaboration at nascent muscle spindles (A-H) or GTOs (H-L) visualized by the expression of PV (white: A, B, D-F, H, I-L) or double-label immunocytochemistry to PV (green) and Egr3 (red: C, G) in hindlimb muscles of E15.5 (A-C), E16.5 (D, E, I, J), E18.5 (H, K, L) and P3 (F, G) wild-type (A-I, K) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant (J, 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: (A, I-L) = 20 m; (B, C, D, E) = 25 m; (F, G) = 40 m; (H) = 80 m.

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 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 (Figure 14C, F, I, L). In contrast, expression of the *Ig-Nrg1* isoform was first detected at E14.5, and between E14.5 and E18.5 was restricted to a subpopulation of DRG neurons (Figure 14B, E, H, K). 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 (Figure 14K, N). 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 (Figure 14K, Q), supporting the idea that the *Ig-Nrg1* isoform is expressed by proprioceptive neurons. Furthermore, Ig-Nrg1 expression was examined in DRG of mice that inherit the previously described *neuregulin-LacZ* fusion allele, *Nrg1*$^{\Delta EGF\text{-}LacZ}$ (Meyer and Birchmeier, 1995; Meyer et al., 1997). In this allele *LacZ* was introduced into exon 6 present in all isoforms. Nevertheless, active β-galactosidase (LacZ) was observed only at sites of type I neuregulin (Ig-Nrg1) expression in heterozygous *Nrg1*$^{\Delta EGF\text{-}LacZ/+}$ embryos (Meyer et al., 1997). Using immunohistochemistry, the expression of Ig-Nrg1 (LacZ) was indeed detected in proprioceptive E17.5 DRG sensory neurons as $LacZ^+$ cells co-expressed the prominent proprioceptive markers PV (Figure 15A-C, Honda 1995; Arber et al., 2000), Er81 (Figure 15D-F, Arber et al., 2000), and Runx3 (Figure 15G-I, Levanon et al., 2002; Inoue et al., 2002), respectively.

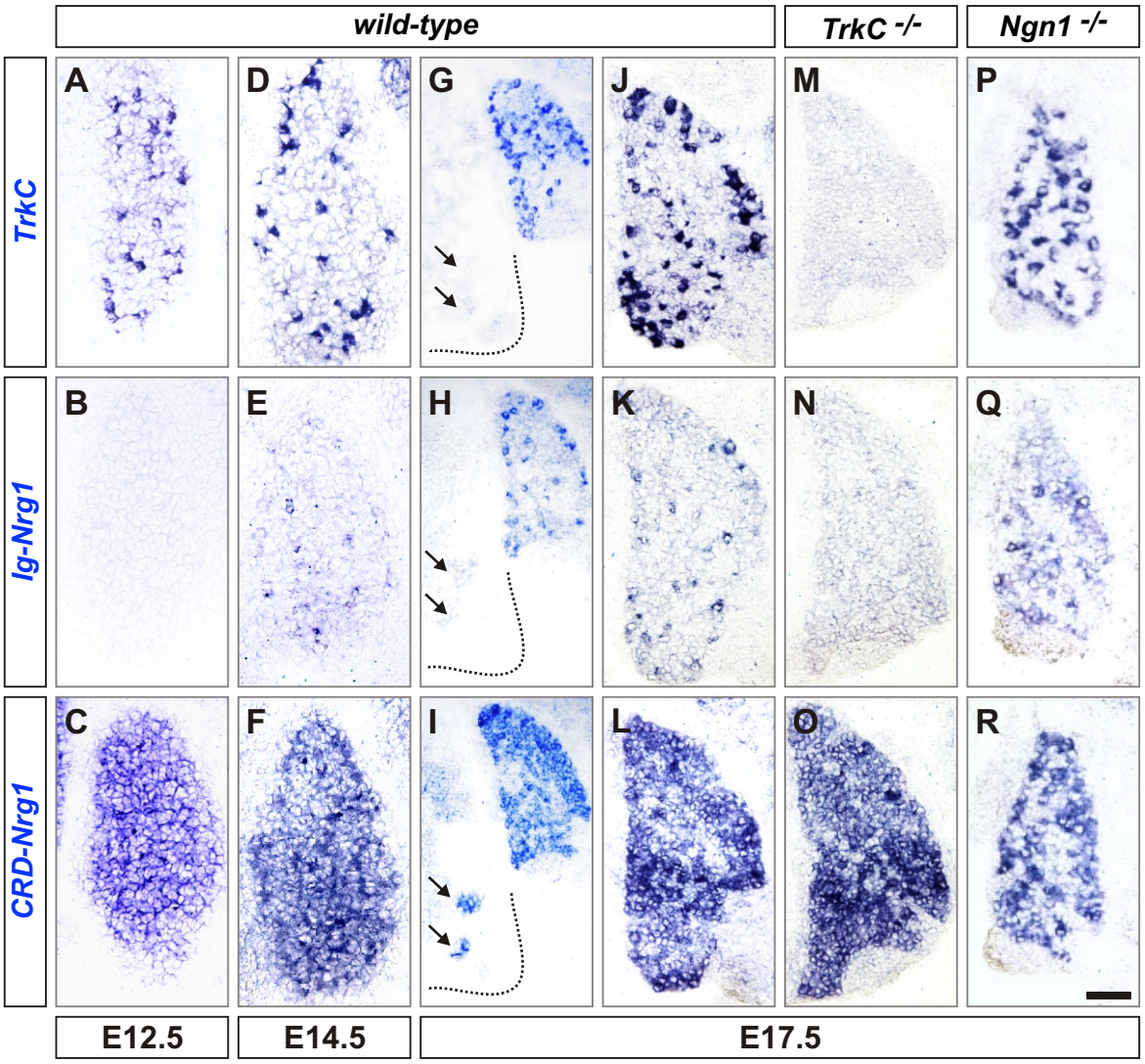

Figure 14. *Ig-Nrg1* but not *CRD-Nrg1* Expression is Restricted to Proprioceptive Afferents. Expression of *TrkC* (A, D, G, J, M, P), *Ig-Nrg1* (B, E, H, K, N, Q) and *CRD-Nrg1* (C, F, I, L, O, R) in lumbar DRGs of wild-type (A-L), *TrkC*$^{-/-}$ (M-O) and *Ngn1*$^{-/-}$ (P-R) embryos at E12.5 (A-C), E14.5 (D-F), and E17.5 (G-R). (G-I) Low-magnification view of DRGs 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: (A-F; P-R) = 60 m; (G-I) = 140 m; (J-O) = 80 m.

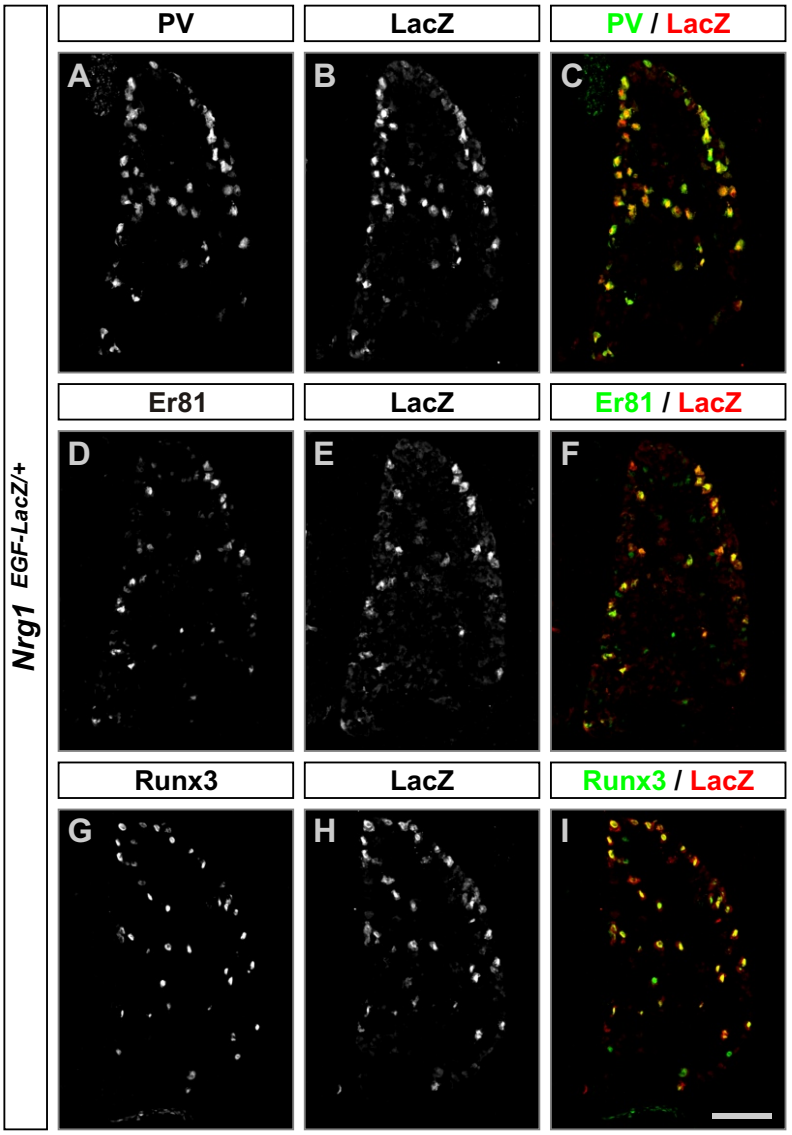

Figure 15. Ig-Nrg1 is Expressed in Proprioceptive Afferents. Expression of PV (white: A; green: C), Er81 (white: D; green: F), Runx3 (white: G; green: I) and LacZ (white: B, E, H; red: C, F, I) in lumbar DRG in *Nrg1* $^{EGF\text{-}LacZ/+}$ heterozygous mice at E17.5. Scale bar: = 110 m.

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 (Figure 14O, R). Together, these findings provide evidence that proprioceptive afferents co-express 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 14I; data not shown), consistent with previous findings (Meyer et al., 1997; Yang et al., 1998). In contrast, much lower levels of expression of *Ig-Nrg1* isoforms were detected in embryonic motor neurons over the same developmental stages (Figure 14H; data not shown; see also 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 by PV^+ proprioceptive nerve endings (Figure 16A-F). 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).

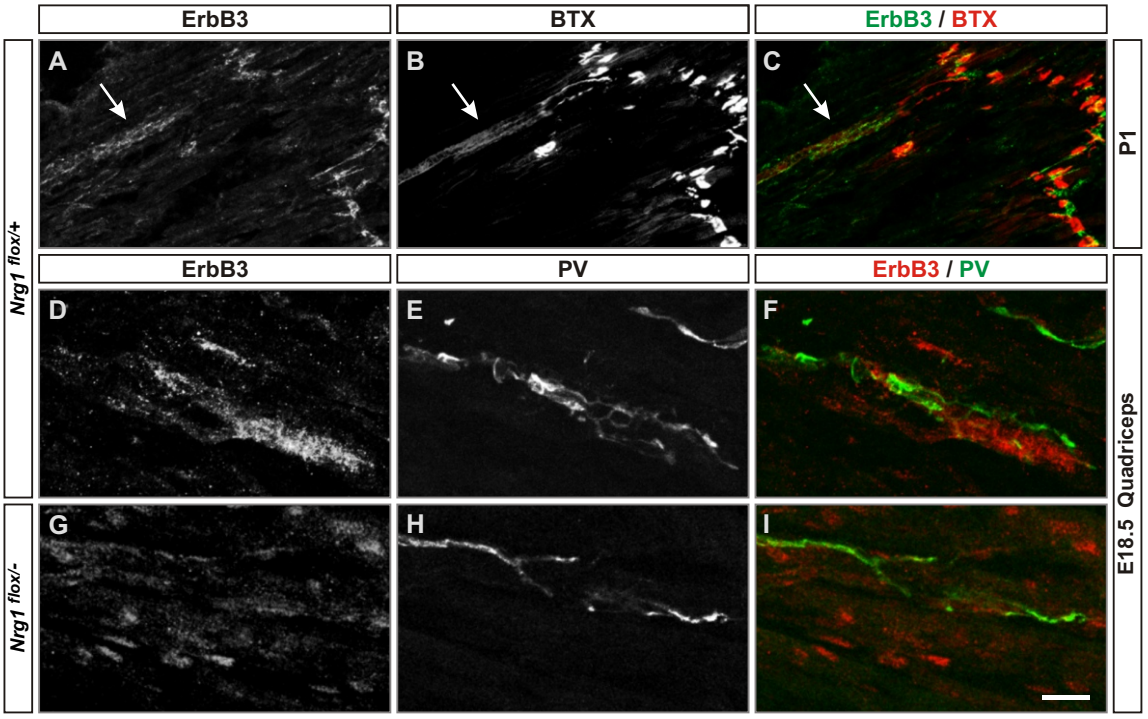

Figure 16. ErbB3 Receptor Expression at Nascent Muscle Spindles. Hindlimb muscles of P1 (A-C) and E18.5 (D-I; quadriceps) wild-type (A-F) and *Isl1*^{CRE}*/Nrg1* $^{flox/-}$ mutant mice (G-I) analyzed by immunocytochemical staining of ErbB3 (white: A, D, G; green: C; red: F, I), AChRs using -bungarotoxin (BTX; white: B; red: C) and PV (white: E, H; green: F, I). Arrows mark nascent muscle spindle. Scale bar: (A-C) = 30 m; (D-I) = 15 m.

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 (*Nrg1* ^{flox}; 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 *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants. The survival and initial differentiation of proprioceptive afferent sensory neurons is not impaired in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants (Figure 17), 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^{+} afferents (Figure 18A-D), and by E18.5, >90% of myofibers on sections adjacent to those containing PV^{+} terminals expressed *Egr3*, *Pea3* and *Erm* (Figure 18I-L). Thus, individual intrafusal muscle fibers are represented consistently in adjacent sections. In contrast, in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants at E16.5 and E18.5, none of the myofibers contacted by PV^{+} axons expressed *Egr3*, *Pea3* and *Erm* (Figure 18E-H, M-P, S). However, expression of *Erm* in the synaptic endplate band of extrafusal muscle fibers contacted by α-motor neurons persisted in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants (Figure 18E, F, M, N). We also detected a lack of accumulation of ErbB3 in myofibers contacted by PV^{+} fibers in these mutants (Figure 16G-I), consistent with the absence of expression of genes expressed selectively by intrafusal muscle fibers in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants.

We next examined whether the loss of muscle spindle differentiation in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants influences the morphology of PV^{+} proprioceptive afferent terminals. In E16.5 hindlimb muscles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 (Figure 19A, E). In *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 (Figure 13A-C; Figure 19E). To determine whether the innervation of intrafusal muscle fibers in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 19B), but in

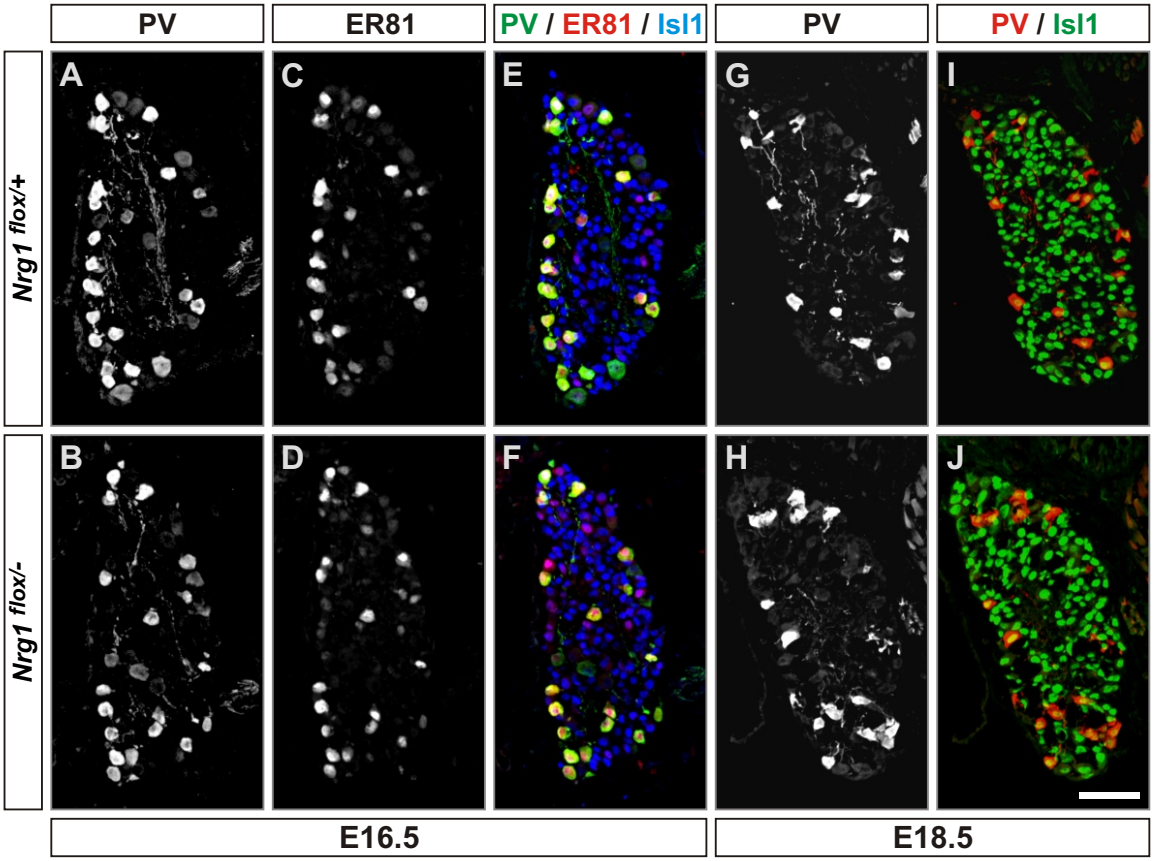

Figure 17. Survival and Gene Expression of Proprioceptive Afferents in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ Mutant Mice. E16.5 (A-F) and E18.5 (G-J) lumbar DRGs of wild-type (A, C, E, G, I) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant (B, D, F, H, J) mice were stained for PV (white in A, B, G, H; green in E, F; red in I, J), ER81 (white in C, D; red in E, F) and Isl1 (blue in E, F; green in I, J). Scale bar = 85 m.

Isl1^{Cre}/*Nrg1* $^{flox/-}$ mutants, PV^{+} proprioceptive endings remained primitive and unbranched (Figure 19F). A similar defect in elaboration of terminal endings was evident when proprioceptive endings were visualized by GAP-43 expression (Figure 19C, D, G, H). In contrast, PV^{+} afferents located at myotendinous regions that prefigure the position of Golgi Tendon Organs (GTOs; Zelena, 1994), possessed a flame-shaped arborization pattern similar to that in wild-type mice, when assayed at E16.5 to E18.5 (Figure 13I-L).

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 *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 cells development in hindlimb muscles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 (Figure 20A, B, D, E, G, H; data not shown; see Experimental Procedures for a likely explanation of the difference in Schwann cell survival in different muscles in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants). Importantly, in our analysis of muscle spindle differentiation, we detected similar defects in dorsal (quadriceps) and ventral (adductor and gracilis) hindlimb muscles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutants. These findings argue strongly that the status of Schwann cell differentiation is unrelated to the process of muscle spindle differentiation, supporting the idea that Nrg1 signaling acts directly on myofibers to initiate muscle spindle differentiation.

Together, these findings provide evidence that the elimination of all isoforms of *Nrg1* from developing DRG blocks the initiation of muscle spindle differentiation.

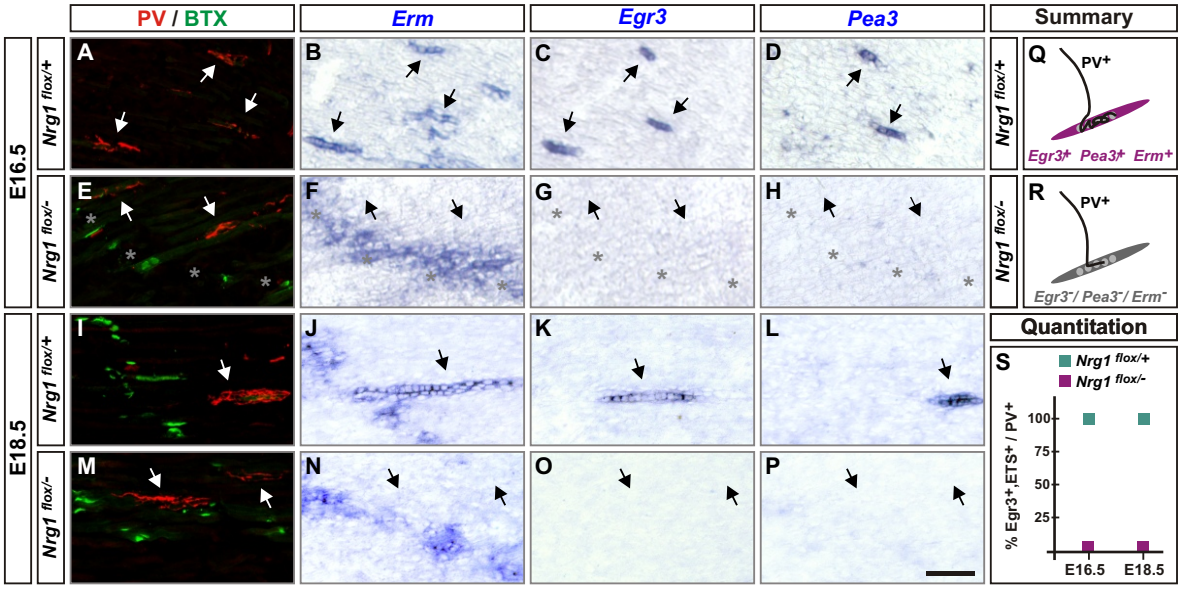

Figure 18. Lack of Induction of *Egr3*, *Pea3* and *Erm* Expression in Intrafusal Muscle Fibers of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ Mutant Mice. (A-P) Analysis of muscle spindle differentiation at E16.5 (A-H) and E18.5 (I-P) in wild-type (A-D, I-L) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant (E-H, M-P) mice. Arrows point to the same muscle spindles on adjacent sections and asterisks (E-H) depict location of synaptic endplate band. (A, E, I, M) Immunocytochemical staining of PV (red) and AChRs using -bungarotoxin (BTX; green). (B, F, J, N) *In situ* hybridization analysis of *Erm* expression. (C, G, K, O) *In situ* hybridization analysis of *Egr3* expression. (D, H, L, P) *In situ* hybridization analysis of *Pea3* expression. (Q, R) Summary diagram of an embryonic muscle spindle in wild-type (Q) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ 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 = 35 m.

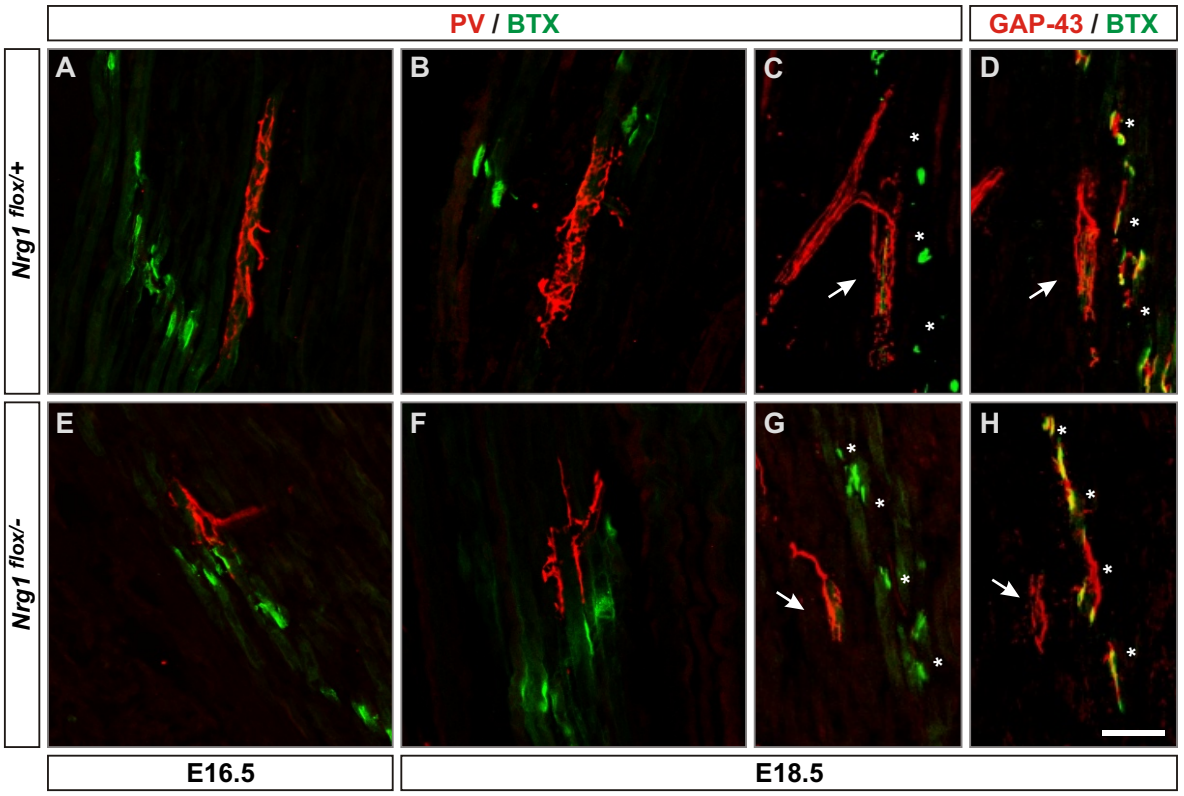

Figure 19. Proprioceptive Afferents at Prospective Muscle Spindles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ Mutant Mice Show Branching Defects. Analysis of muscle spindle innervation (arrows mark same spindle on adjacent sections) in hindlimb muscles of E16.5 (A, E) and E18.5 (B-D, F-H), wild-type (A-D) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant mice (E-H) by immunocytochemical staining of PV (red; A-C, E-G) or GAP-43 (red; D, H on adjacent sections to C, G) and AChRs using -bungarotoxin (BTX; green). Asterisks mark location of synaptic endplate band. Scale bar = (A, B, E, F) = 20 m; (C, D, G, H) = 30 m.

An Early Defect in Muscle Spindle Differentiation in Mice Lacking ErbB2 in Skeletal Muscle Fibers

To induce the cellular signaling transduction cascade(s), neuregulins bind to ErbB receptor complexes located at the plasma membrane (Burden and Yarden, 1997; Buonanno and Fischbach, 2001). A crucial ErbB subunit that mediates the phosphorylation and activation of downstream effectors is ErbB2 in a complex together with either ErbB3 or ErbB4 (Burden and Yarden, 1997; Buonanno and Fischbach, 2001). A conditional ErbB2 allele allowed us to analyze muscle spindle differentiation in mice lacking functional ErbB receptor complexes selectively in skeletal muscles (*HSA^{Cre}/ErbB2 $^{flox/-}$*; Leu et al., 2003). The phenotype observed in *HSA^{Cre}/ErbB2 $^{flox/-}$* mutants with respect to muscle spindle differentiation was identical to the one described for *Isl1^{Cre}/Nrg1 $^{flox/-}$* mutants as assessed by the lack of expression of *Egr3*, *Pea3* and *Erm* in embryonic hindlimb muscles in *HSA^{Cre}/ErbB2 $^{flox/-}$* mutant mice at E16.5 (Figure 21, data not shown). In addition to absent intrafusal gene expression in *HSA^{Cre}/ErbB2 $^{flox/-}$* mutants, Ia proprioceptive afferents failed to establish the characteristic annulospiral end terminals in the central region of intrafusal muscle fibers. Despite initial contacts were established with muscle fibers, maturation of the PV^{+} Ia afferent endings to fully differentiated end terminals was severely affected in *HSA^{Cre}/ErbB2 $^{flox/-}$* embryos (Figure 22) similar to the primitive Ia afferent endings detected in hindlimb muscles of *Isl1^{Cre}/Nrg1 $^{flox/-}$* mutants.

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 differentiation. Analysis of hindlimb muscles of *CRD-Nrg1* mutant mice at E16.5 revealed that expression of *Egr3*, *Pea3* and *Erm* was initiated in muscle spindles in a manner indistinguishable from that observed in wild-type embryos (Figure 23B-D, F-H). In addition, PV^{+} proprioceptive axons had contacted individual myofibers and elaborated terminal branches in the central region of nascent intrafusal muscle fibers (Figure 23E). The pattern of PV^{+} afferent innervation at muscle spindles in *CRD-Nrg1* mutants could not be distinguished from that in wild-type mice (Figure 23A). These results indicate that *CRD-Nrg1* is not required for muscle spindle differentiation and imply that the *Ig-Nrg1* isoforms selectively expressed by proprioceptive sensory neurons are sufficient to induce muscle spindle differentiation. Moreover, in *CRD-Nrg1 $^{-/-}$* mutant mice we detected no intramuscular Schwann cells in dorsal or ventral hindlimb muscles (Figure 20C, F, I;

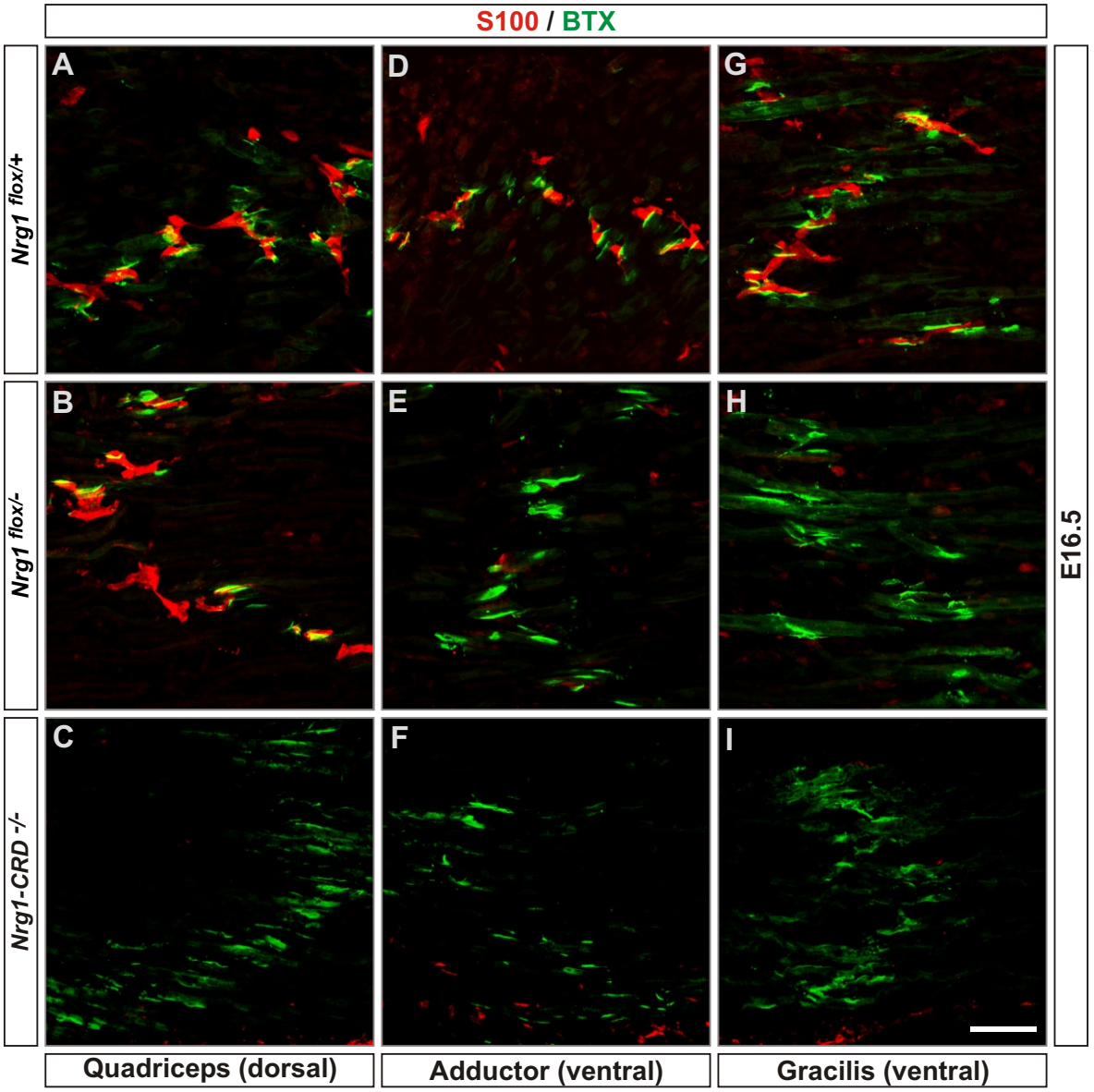

Figure 20. Schwann Cell Defects in Hindlimb Muscles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ and *CRD-Nrg1*$^{-/-}$ Mutant Mice. Double-label immunocytochemistry of S100^{+} Schwann cells (red) and clusters of AChRs with -bungarotoxin (BTX; green) in quadriceps (A-C), adductor (D-F) and gracilis (G-I) muscles of E16.5 hindlimbs of wild-type (A, D, G), *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant (B, E, H) and *CRD-Nrg1*$^{-/-}$ (C, F, I) mice. Note selective absence of Schwann cells in adductor and gracilis muscles of *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant mice and absence of Schwann cells in all muscles of *CRD-Nrg1*$^{-/-}$ mutant mice. A similar phenotype was already observed at E14.5 to E15.5 (data not shown). Scale bar = 40 m.

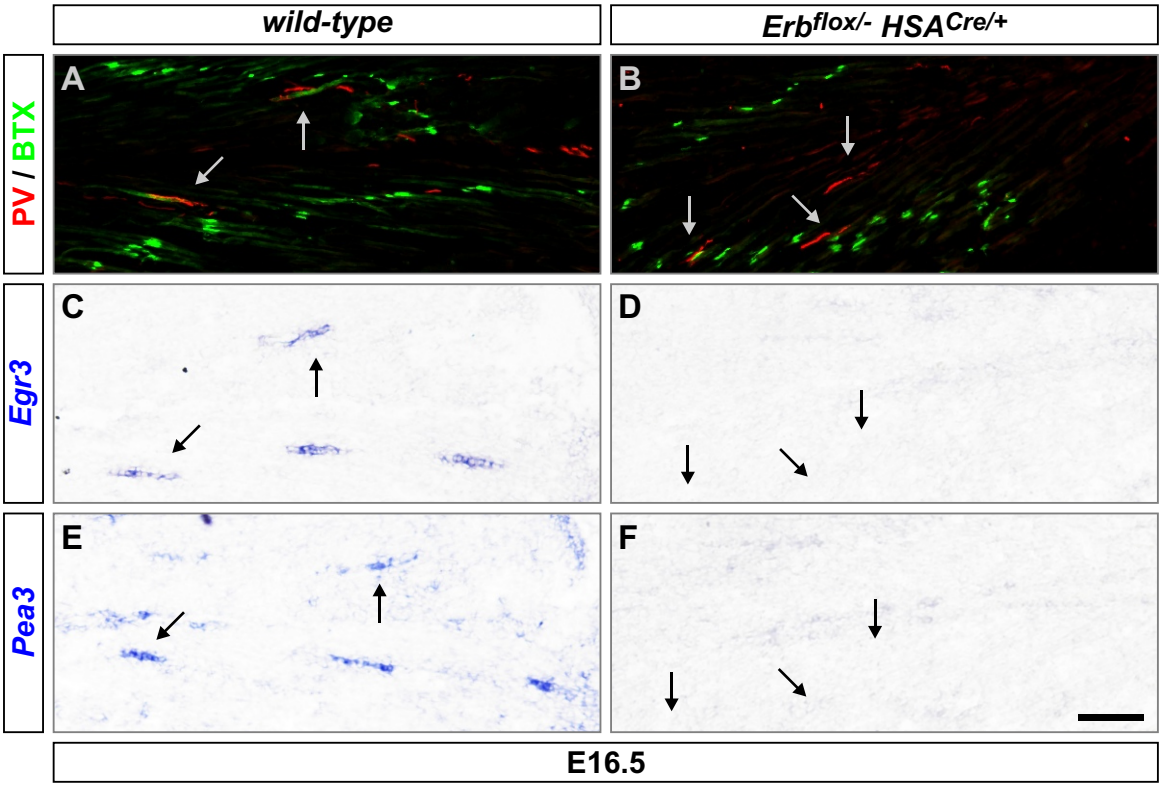

Figure 21. Lack of Induction of *Egr3* and *Pea3* Expression in Intrafusal Muscle Fibers of *HSA*^{Cre}/*ErbB2* $^{flox/-}$ Mutant Mice. (A-F) Analysis of muscle spindle differentiation at E16.5 in wild-type (A, C, E) and *HSA*^{Cre}/*ErbB2* $^{flox/-}$ mutant (B, D, F) mice. Arrows point to the same muscle spindles on adjacent sections. (A, B) Immunocytochemical staining of PV (red) and AChRs using -bungarotoxin (BTX; green). (C, D) *In situ* hybridization analysis of *Egr3* expression. (E, F) *In situ* hybridization analysis of *Pea3* expression. Scale bar = 45 m.

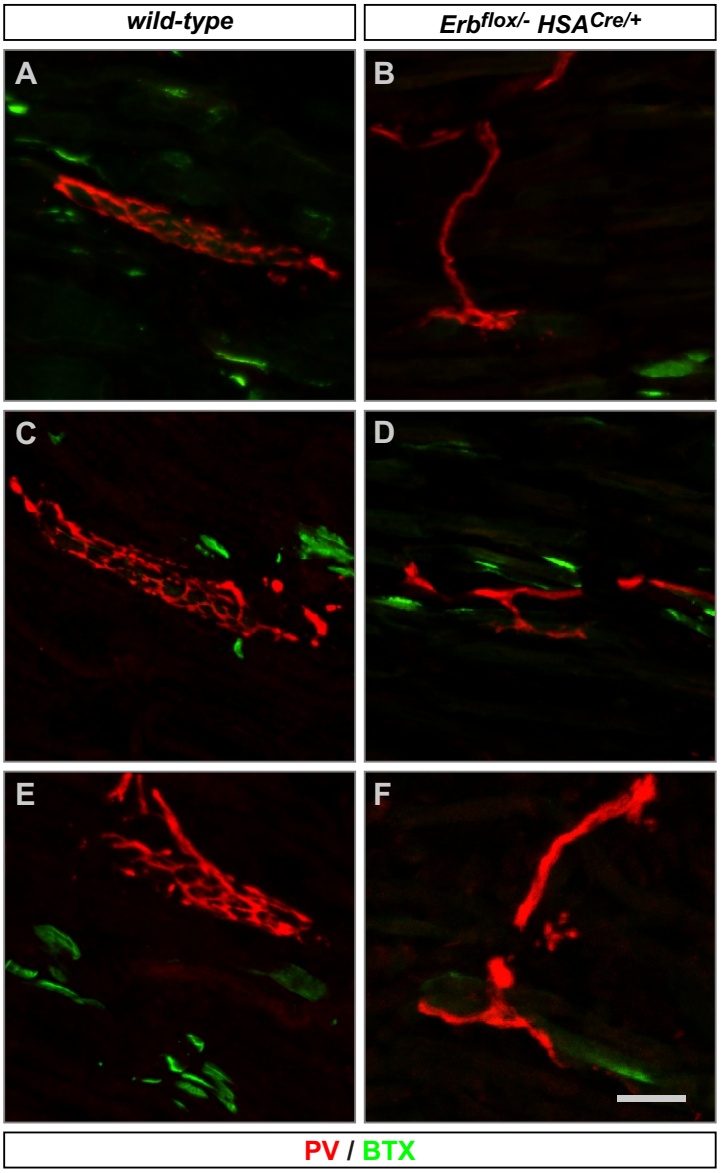

Figure 22. Proprioceptive Afferents at Prospective Muscle Spindles of *HSA*^{Cre}/*ErbB2* $^{flox/-}$ Mutant Mice Show Branching Defects. Analysis of muscle spindle innervation in hindlimb muscles of E16.5 wild-type (A, C, E) and *HSA*^{Cre}/*ErbB2* $^{flox/-}$ mutant mice (B, D, F) by immunocytochemical staining of PV (red) and AChRs using -bungarotoxin (BTX; green). Scale bar = (A, B) = 20 m; (C, D, E) = 15 m; (F) = 10 m.

Wolpowitz et al., 2000), providing further evidence for a dissociation in the fate of Schwann cells and the differentiation of muscle spindles.

A comparative summary with respect to the state of muscle spindle differentiation in mutant mice carrying either of the two different *Nrg1* alleles or an *ErbB2* mutation is shown in Figure 24.

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 $Isl1^{Cre}/Nrg1^{flox/-}$ 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 $Isl1^{Cre}/Nrg1^{flox/-}$ mutant mice (Figure 25). 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.

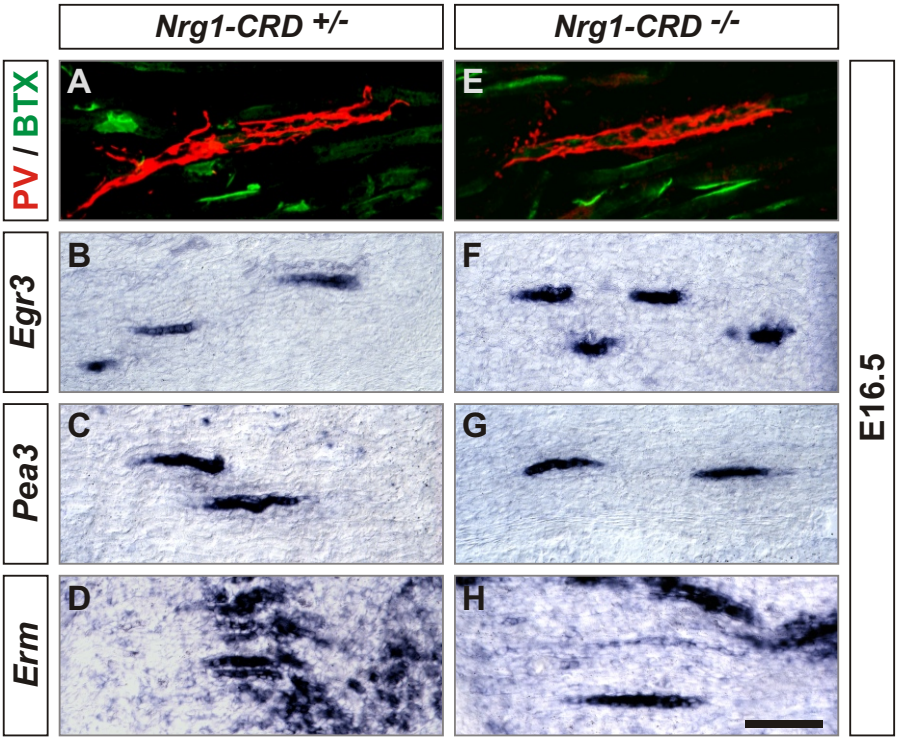

Figure 23. *CRD-Nrg1* Mutant Mice Do not Exhibit a Defect in Early Muscle Spindle Differentiation. Immunocytochemical detection of PV^{+} proprioceptive afferents at developing muscle spindles (A, E), AChRs using -bungarotoxin (A, E; BTX; green), and *in situ* hybridization analysis of *Egr3* (B, F), *Pea3* (C, G), and *Erm* (D, H) in intrafusal muscle fibers of E16.5 hindlimb muscles of wild-type (A-D) and *CRD-Nrg1*$^{-/-}$ (E-H) mice. Scale bar: (A, E) = 20 m; (B-D; F-H) = 65 m.

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 26). Our findings show that *Ig-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: (i) the early expression and function of transcription factors induced in nascent intrafusal muscle fibers; (ii) the role of *Nrg1* isoforms in skeletal muscle fiber differentiation; (iii) 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

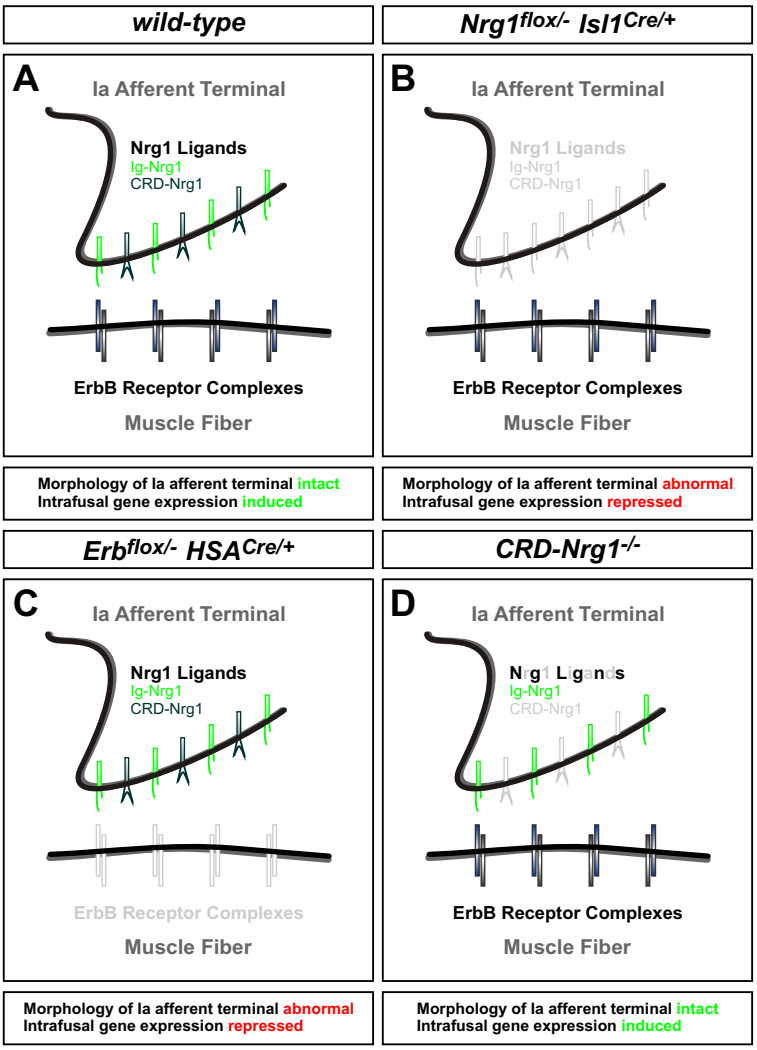

Figure 24. Summary of Muscle Spindle Phenotypes in Wild-type, Neuronal *Nrg1*, and Skeletal *ErbB2* Mutations. (A-D) Schematic summary diagrams of the Ia afferent terminal - intrafusal muscle fiber interface in wild-type (A), *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ (B), *HSA*^{Cre}/*ErbB2* $^{flox/-}$ (C), and *CRD-Nrg1*$^{-/-}$ (D) animals. Ig-Nrg1 (green) and CRD-Nrg1 (black) ligands expressed by Ia afferents and ErbB receptor complexes (blue and black) expressed in intrafusal muscle fibers are indicated. Ablation of either Nrg1 ligands or ErbB2 receptor subunits is indicated by light grey color of the respective molecules. Muscle spindle differentiation is blocked in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ (B) and *HSA*^{Cre}/*ErbB2* $^{flox/-}$ (C) but not in *CRD-Nrg1*$^{-/-}$ (D) mutant animals in comparison to wild-type (A) as assessed by intrafusal gene expression and Ia afferent terminal branching morphology.

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 co-expression 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 muscle spindle differentiation. Nevertheless, our findings imply that such transcription factors are induced in intrafusal muscle fibers in response to Nrg1 signaling.

Nrg1 Isoforms and Muscle Spindle Differentiation

The early onset of expression of *Egr3*, *Pea3* and *Erm* by intrafusal muscle fibers has provided a set of molecular markers to assay candidate signaling molecules that might mediate the neurally-evoked induction of muscle spindle differentiation. Several lines of evidence support the view that Nrg1 functions as a critical mediator of this proprioceptive afferent-derived inductive signal, and suggest that the Ig isoforms of Nrg1 are the relevant inducers of muscle spindle differentiation.

First, expression of *Ig-Nrg1* isoforms of Nrg1 is largely restricted to proprioceptive afferent neurons, and is detected only at very much lower levels by cutaneous sensory neurons and by motor neurons. In contrast, the expression of *CRD-Nrg1* isoforms is detected in most or all DRG neurons, and at high levels by motor neurons. 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 slightly lower than the total number of $TrkC^+$ proprioceptive sensory neurons, raising the possibility that only a subset of proprioceptors express Ig-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 Ib afferents.

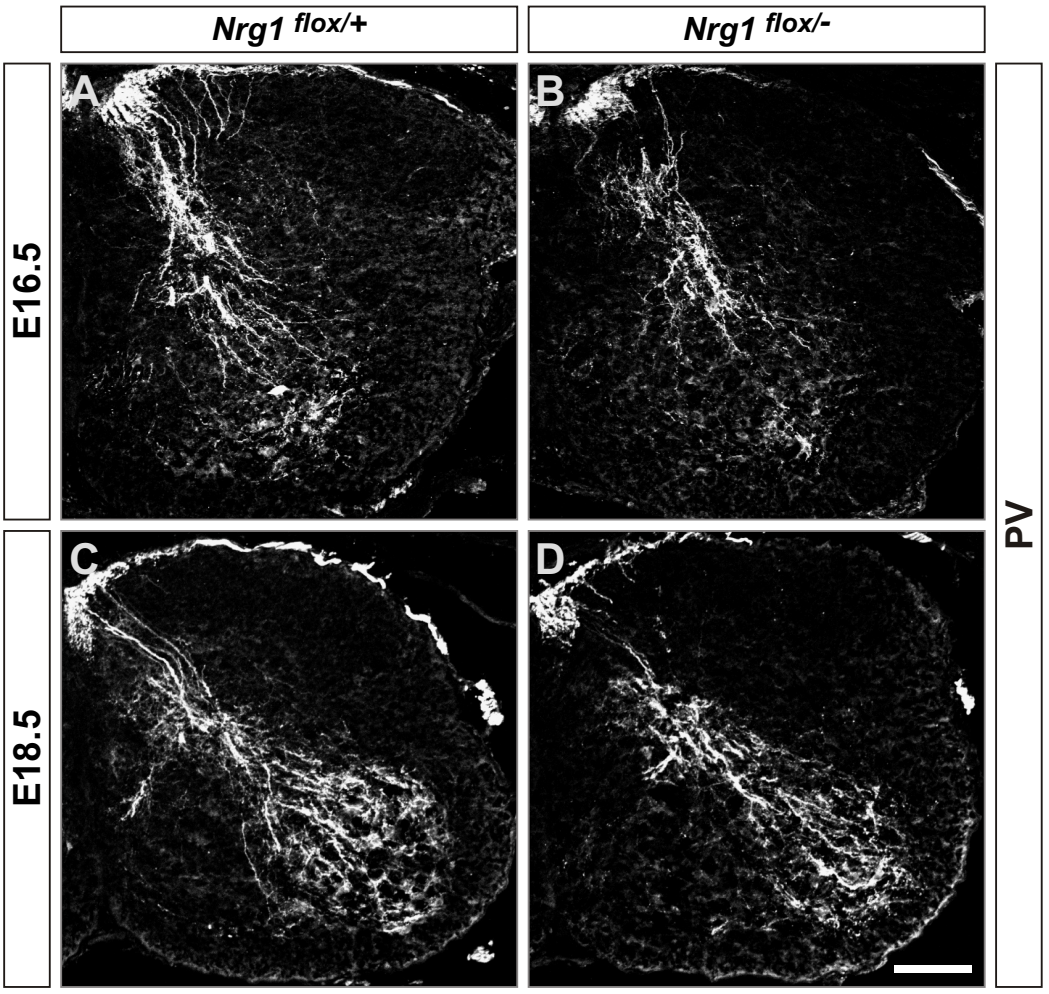

Figure 25. No Defect in Central Projections of Proprioceptive Afferents in *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ Mutant Mice. Central projections of proprioceptive afferents in E16.5 (A, B) and E18.5 (C, D) lumbar spinal cord of wild-type (A, C) and *Isl1*^{Cre}/*Nrg1* $^{flox/-}$ mutant (B, D) mice were traced by expression of PV. Scale bar = 115 m.

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 isoforms of Nrg1, are sufficient, and CRD-containing isoforms 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.

The idea that the *Ig-Nrg1* rather than the *CRD-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 co-expressed 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

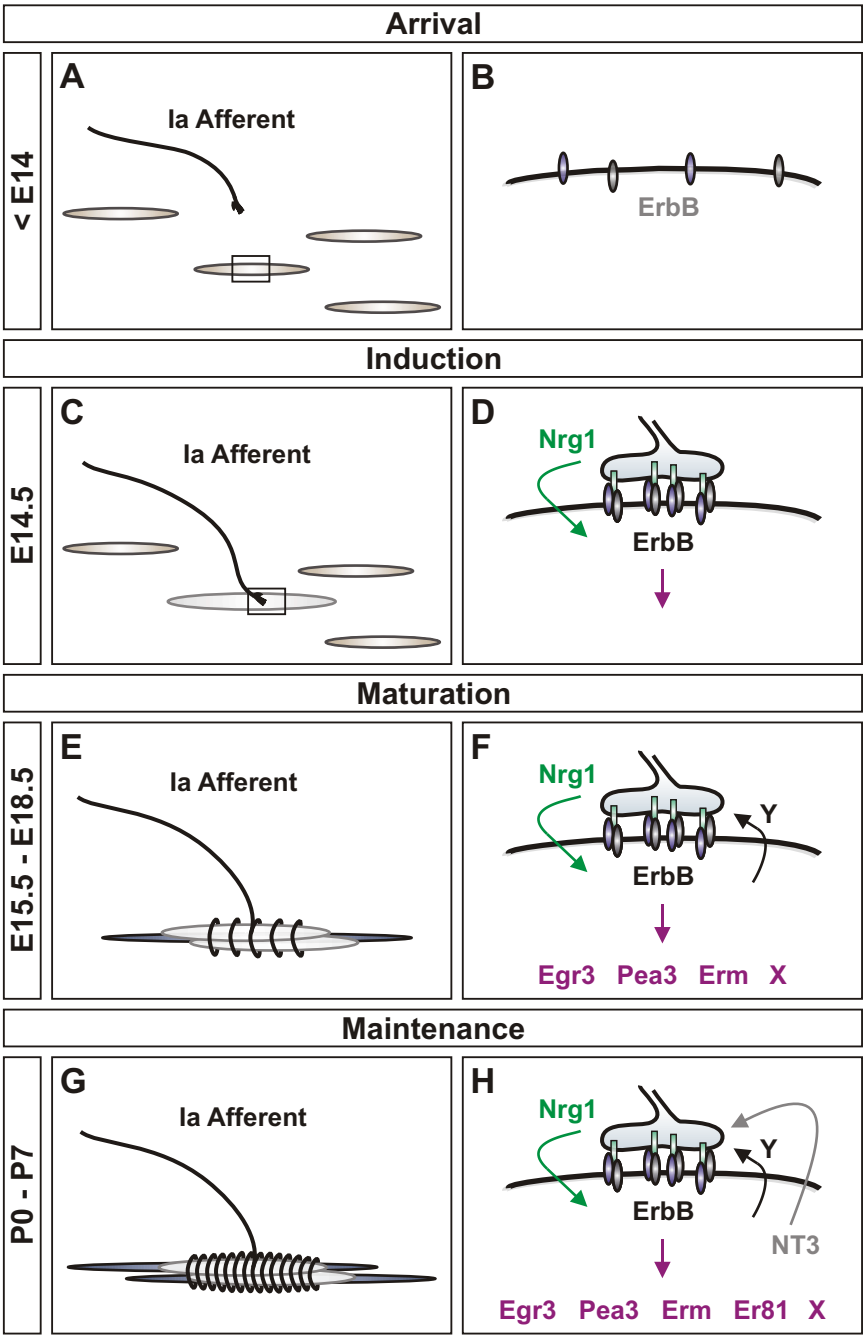

Figure 26. *Nrg1* Expression by Proprioceptive Afferents is Critical for the Initiation of Muscle Spindle Differentiation. Summary diagrams of the developmental transition of an unspecified myotube to a fully differentiated muscle spindle (A, C, E, G) and accompanying molecular signals involved in the specification of intrafusal muscle fibers (B, D, F, H). (A, B) Before invasion of Ia afferents into a muscle, intrafusal and extrafusal muscle fibers cannot be distinguished molecularly and both express ErbB receptors. (C, D) Ia afferents expressing *Nrg1* contact intrafusal muscle fibers, activate ErbB receptor complexes (purple arrow in D) and initiate muscle spindle differentiation. (E, F) After initial contact of Ia afferents with prospective intrafusal muscle fibers, proprioceptive afferents branch extensively (E) and signaling events downstream of Nrg1/ErbB-receptor complexes result in the induction of the transcription factors *Egr3*, *Pea3*, *Erm*, and factor(s) X (F). A retrograde signal Y from the intrafusal muscle fibers may promote elaboration of annulospiral endings of proprioceptive afferents. (G, 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 Ia afferents with motor neurons (grey arrow).

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). Furthermore, the findings as a result of the phenotypic analysis of $HSA^{Cre}/ErbB2^{flox/-}$ mutant mice provides clear direct evidence that ErbB2 signaling mediates the embryonic role of Nrg1 signaling in early muscle spindle differentiation.

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 post-synaptic 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 et al., 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 post-synaptic 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 and ErbB2 Mutant Mice

Neuronal *Nrg1* and skeletal muscle *ErbB2* 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 $Isl1^{Cre}/Nrg1^{flox/-}$ and $HSA^{Cre}/ErbB2^{flox/-}$ 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 (Zelena, 1994).

These observations raise the question of how the loss of neuronally-derived Nrg1 signaling regulates the morphology of proprioceptive afferent terminals. Four possibilities can be considered. First, neuronally-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 *Isl1*^{Cre}/*Nrg1*$^{flox/-}$ 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 (Bao et al., Soc. Neurosci. Abstract *27*, 2001), 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 (Bao et al., Soc. Neurosci. Abstract *27*, 2001), 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 *Erm*.

One secreted signaling molecule known to be expressed by developing muscle spindles is NT-3 (Copray and Brouwer, 1994; Chen et al., 2002b). Reciprocal cell-cell interactions involving Nrg1 and NT-3 have been documented between neuroblasts and non-neuronal 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 Ia 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 *Er81* from muscle spindles and proprioceptive sensory neurons results in a marked defect in the projection of group Ia 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., 2002b; 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 non-neural 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 other organisms.

Chapter 6

DRG Neurons Exhibit a Developmental Switch in Response to ETS Transcription Factor Signaling

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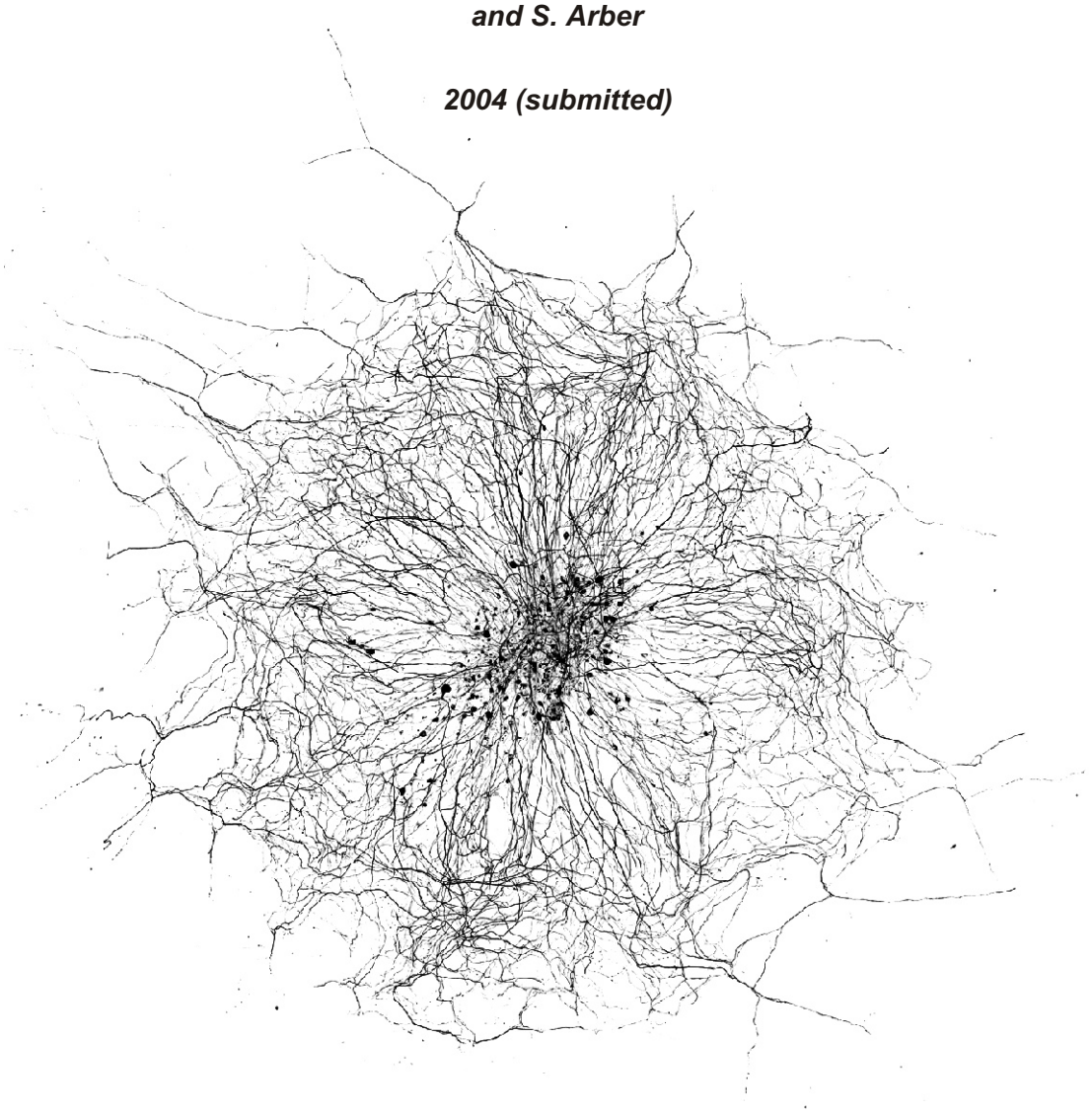
2004 (submitted)



SUMMARY

Two ETS transcription factors of the Pea3 subfamily are induced in subpopulations of dorsal root ganglion (DRG) sensory and spinal motor neurons by target-derived factors. Their expression is essential to control late aspects of neuronal differentiation such as target invasion and branching. Here, we show that upregulation of ETS transcription factor signaling specifically at a late stage of proprioceptive DRG sensory neuron differentiation is important to fulfill an appropriate developmental function. We provide evidence that premature ETS signaling interferes with establishment of neuronal projections, acquisition of terminal neuronal traits and dependence on neurotrophic support. In contrast, late expression of the identical ETS transcriptional regulator in the same neuronal lineage can substitute for ETS gene function and promote neuronal differentiation. Together, these findings suggest that DRG sensory neurons undergo a temporal developmental switch, revealed by distinct responses to ETS transcription factor signaling at sequential steps of neuronal maturation.

INTRODUCTION

Neuronal differentiation is a protracted process during which newly generated neurons go through distinct cellular and molecular steps of maturation. The initial phase of long-distance axon outgrowth and pathfinding of axons is followed by a switch in their developmental program in the vicinity of the target region, when axons shift their growth program to promote target invasion, branching and formation of synapses within the target region. Current evidence suggests that many important traits of neuronal character are already acquired at progenitor cell stages, translating into distinct early post-mitotic neuronal identities (Edlund and Jessell, 1999). In contrast, it is less clear how crucial the timely acquisition of additional neuronal subtype specific features explicitly at later stages is for neuronal maturation, and thus ultimately for the assembly and maturation of neuronal circuits.

The differentiation of dorsal root ganglia (DRG) sensory neurons has been studied extensively with respect to early inductive events involved in initial neuronal fate acquisition (Anderson et al., 1997; Anderson, 2000; Bertrand et al., 2002; Knecht and Bronner-Fraser, 2002), as well as late target-derived factors controlling neuronal survival (Huang and Reichardt, 2001). Early steps in the

differentiation of DRG sensory neurons appear to occur in the absence of interactions with the peripheral targets. Successive waves of DRG neurogenesis are controlled by the action of the basic helix loop helix transcription factors Ngn1 and Ngn2 (Perez et al., 1999; Ma et al., 1999) and give rise to early postmitotic DRG neurons. Subsequently, the POU-domain transcription factor Brn3A is required to promote initial sensory axon outgrowth towards peripheral targets (Eng et al., 2001). Once DRG sensory axons reach the vicinity of their peripheral targets, neuronal survival is tightly regulated by the local availability of different neurotrophic factors signaling through neurotrophin receptors expressed by distinct populations of DRG sensory neurons (Huang and Reichardt, 2001).

Several lines of evidence suggest that target-derived neurotrophic factors are also involved in regulating late aspects of neuronal differentiation independent of a role for survival (McAllister et al., 1999; Bibel and Barde, 2000; Mendell et al., 2001; Markus et al., 2002a). Adult DRG sensory neurons cultured in the absence of neurotrophic factors survive without NGF, but require NGF for the expression of aspects of neurotransmitter phenotype, including the neuropeptides substance P and calcitonin gene-related peptide (Lindsay and Harmar, 1989). Moreover, recent genetic experiments have addressed the survival-independent role of neurotrophic factors during development *in vivo* by exploiting strains of mice coincidently mutant in neurotrophins or their receptors and the proapoptotic gene *Bax* (Patel et al., 2000; Patel et al., 2003). These experiments have shown that neurotrophin signaling plays multiple roles during late neuronal differentiation including the acquisition of peptidergic traits in nociceptive DRG neurons and the control of target innervation (Patel et al., 2000; Patel et al., 2003).

Integration and interpretation of axon guidance signals encountered by axons occurs mostly locally at the growth cone (Yu and Bargmann, 2001; Dickson 2002; Huber et al., 2003). In contrast, target-derived factors involved in the acquisition of late neuronal properties are thought to act retrogradely in the cell bodies where induced downstream signaling cascades influence or switch programs of neuronal differentiation by specifically intersecting with transcriptional programs established at earlier developmental steps. The molecular mechanisms, by which these interactions between target region and cell bodies occur, are only beginning to be revealed.

Surprisingly, distinct transcriptional programs are not only initiated at cell cycle exit, but recent results suggest that induction of additional transcriptional programs can still occur days after neurons exit the cell cycle. Notably, peripheral neurotrophic signals have been shown to induce expression of the ETS transcription factors (Sharrocks, 2001) Er81 and Pea3 in distinct subpopulations of DRG sensory neurons and motor neuron pools several days after these neurons

become postmitotic (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002; Haase et al., 2002; Patel et al., 2003). Er81 and Pea3 control distinct late aspects of spinal monosynaptic circuit assembly, and an important role for Er81 in proprioceptive DRG neurons and for Pea3 in subpopulations of motor neurons has been described (Arber et al., 2000; Livet et al., 2002). The induction of Er81 in proprioceptive afferents is mediated by peripheral neurotrophin 3 (NT-3; Patel et al., 2003). In the absence of *Er81,* or in *NT-3/Bax* mutant mice, Ia proprioceptive afferents fail to invade the ventral spinal cord and thus fail to make synaptic connections with motor neurons (Arber et al., 2000; Patel et al., 2003). Moreover, Pea3 expression in motor neurons controls target invasion in the periphery and the induction of Pea3 in motor neurons is mediated by peripherally derived Glial cell line-derived neurotrophic factor (GDNF; Haase et al., 2002; Livet et al., 2002).

Together, these experiments show that Er81 and Pea3 control specifically late aspects of neuronal differentiation in specific neuronal lineages, at a developmental time when DRG sensory neurons and motor neurons become dependent on neurotrophic support for survival and switch their growth program from axonal elongation to invasion and branching within the target region. These findings thus provide a potential mechanism by which late acquisition of neuronal traits are superimposed on neurons assigned to specific neuronal lineages at earlier developmental stages and suggest that temporally controlled induction of Er81 and Pea3 in defined neuronal subpopulations might be required to specifically control such late aspects of neuronal differentiation. However, these studies have not addressed whether closely related ETS family members can fulfill similar functions in the same neuronal population, nor have they addressed the necessity for a late target-induced onset of ETS transcription factor expression within postmitotic DRG neurons.

In this study, we have addressed the respective contribution of Er81 and Pea3 for the establishment of Ia proprioceptive afferent projections and the requirement for an appropriate temporal onset of ETS transcription factor signaling in DRG sensory neurons. We show that the function of *Er81* in controlling Ia proprioceptive afferent projections cannot be substituted for by the closely related transcription factor *Pea3* whereas *EWS-Pea3*, a fusion product of *Pea3* with stronger transcriptional transactivation potential than *Pea3*, can substitute for *Er81* function when expressed in proprioceptive afferents from the time of onset of *Er81* expression. In contrast, expression of *EWS-Pea3* in early postmitotic DRG neurons leads to neuronal differentiation characterized by neurotrophin-independent neurite outgrowth and inappropriate gene expression. Such gene expression changes are not observed when *EWS-Pea3* is expressed in proprioceptive neurons at a developmental

time when *Er81* is normally expressed. Together, these findings suggest that late target-induced expression of specific transcription factors in postmitotic neurons is critical to control late neuronal maturation and circuit assembly through appropriate activation of downstream target genes.

RESULTS

EWS-Pea3 but not Pea3 can Replace Er81 Function in Controlling Ia Afferent Projections

We first wanted to assess whether the function of Er81 within proprioceptive afferents to direct projections into the ventral spinal cord can be substituted for by the highly homologous ETS transcription factor Pea3. Several ETS transcription factors including Pea3 have been shown to occur as break-point fusion products between the amino-terminal domain of the Ewing sarcoma (EWS) gene and the corresponding ETS-DNA binding domains (Urano et al., 1996; Arvand and Denny; 2001). Previous studies have suggested that EWS-ETS fusion products may harbor a higher intrinsic transactivation potential than the native ETS transcription factors themselves thereby contributing to their transforming potential during Ewing sarcoma tumorigenesis (May et al., 1993; Ohno et al., 1993; De Alava and Gerald, 2000). These findings prompted us to first compare the relative transactivation potential of the ETS transcription factor Pea3 to the fusion product EWS-Pea3 using a luciferase enzyme-based cell culture transfection assay in COS-7 cells. As a reporter plasmid, we designed a vector containing an array of five Pea3 subfamily consensus ETS DNA-binding sites (GCCGGAAGC; Mo et al., 1998; Bojovic and Hassell., 2001) upstream of a minimal thymidine kinase promoter to drive expression of luciferase (Figure 27A). Cotransfection of Pea3 activated expression of luciferase ~10 fold over baseline levels ($n \geq 7$; 10.7 ± 1.5; Figure 27A), whereas activation of expression after transfection with EWS-Pea3 led to a stronger, ~20 fold activation of luciferase over baseline levels ($n \geq 7$; 20.3 ± 2.7; Figure 27A). Introduction of point mutations into the ETS binding sites within the reporter plasmid abolished the transactivation potential of EWS-Pea3 to base-line levels (data not shown). Together, these findings show that both Pea3 and EWS-Pea3 activate a minimal reporter plasmid in an ETS DNA-binding site dependent manner, but that in these assays, EWS-Pea3 possesses a higher intrinsic transactivation potential than Pea3.

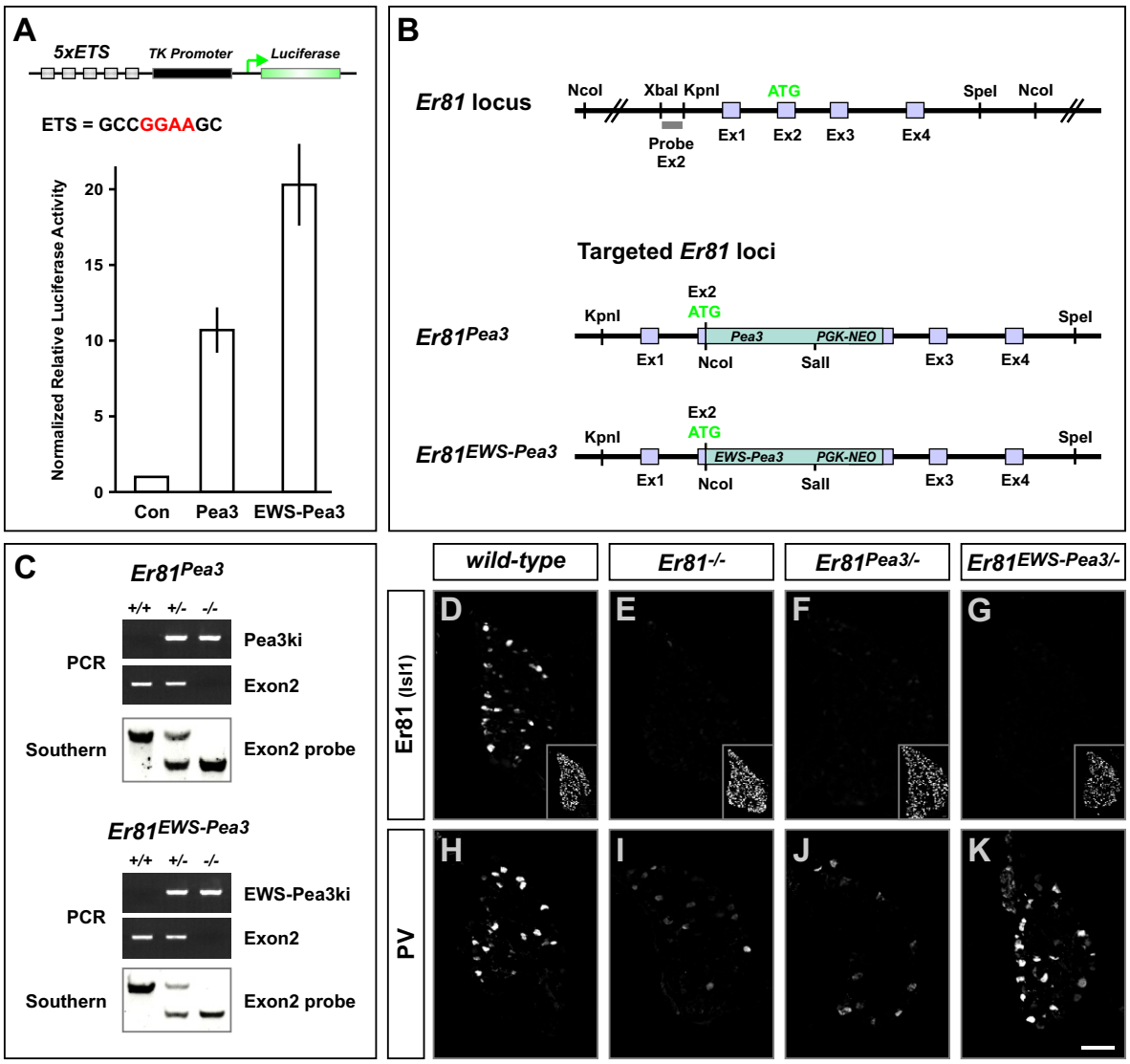


Figure 27. Replacement of *Er81* by *Pea3* and *EWS-Pea3*. (A) Transcriptional transactivation of luciferase expression from a minimal reporter construct containing five consensus ETS DNA binding sites (GCCGGAAGC; Mo et al., 1998; Bojovic and Hassell, 2001) and a minimal TK promoter upon transient transfection of Pea3 or EWS-Pea3 (relative luciferase activity normalized to control; n≥7 values from 3 independent experiments). (B) Generation of $Er81^{Pea3}$ and $Er81^{EWS\text{-}Pea3}$ mutant mice. (Top panel) Organization of the *Er81* genomic locus in the region targeted by homologous recombination in analogy to Arber et al., 2000. Exons 1-4 are shown as light blue boxes and the *Er81* start codon in exon 2 is indicated as ATG. The probe used to detect homologous recombination is shown as a grey box. (Middle; bottom panels) Replacement of *Er81* by *Pea3* (middle) or *EWS-Pea3* (bottom) through the integration of *Pea3* or *EWS-Pea3* in frame with the endogenous start codon of the *Er81* locus in exon 2 (in analogy to Arber et al., 2000). (C) Southern blot and PCR analysis of $Er81^{Pea3}$ and $Er81^{EWS\text{-}Pea3}$ wild-type (+/+), heterozygous (+/-) and homozygous (-/-) genomic DNA to detect the mutant allele. PCR primer pairs Pea3ki and EWS-Pea3ki were used to detect specifically the recombined alleles and a primer pair in exon2 was used to detect the presence of the wild-type allele (Arber et al., 2000). (D-G) Analysis of Er81 expression in lumbar DRG neurons of E16.5 wild-type (D), $Er81^{-/-}$ (E), $Er81^{Pea3/-}$ (F), $Er81^{EWS\text{-}Pea3/-}$ (G) mutant embryos. Inset in lower right corner of each panel shows Islet1 (Isl1) expression in the respective DRG. (H-K) PV expression in lumbar DRG of E16.5 wild-type (H), $Er81^{-/-}$ (I), $Er81^{Pea3/-}$ (J), $Er81^{EWS\text{-}Pea3/-}$ (K) embryos. Confocal scans were performed with equal gain intensity. Scale bar: 80 m.

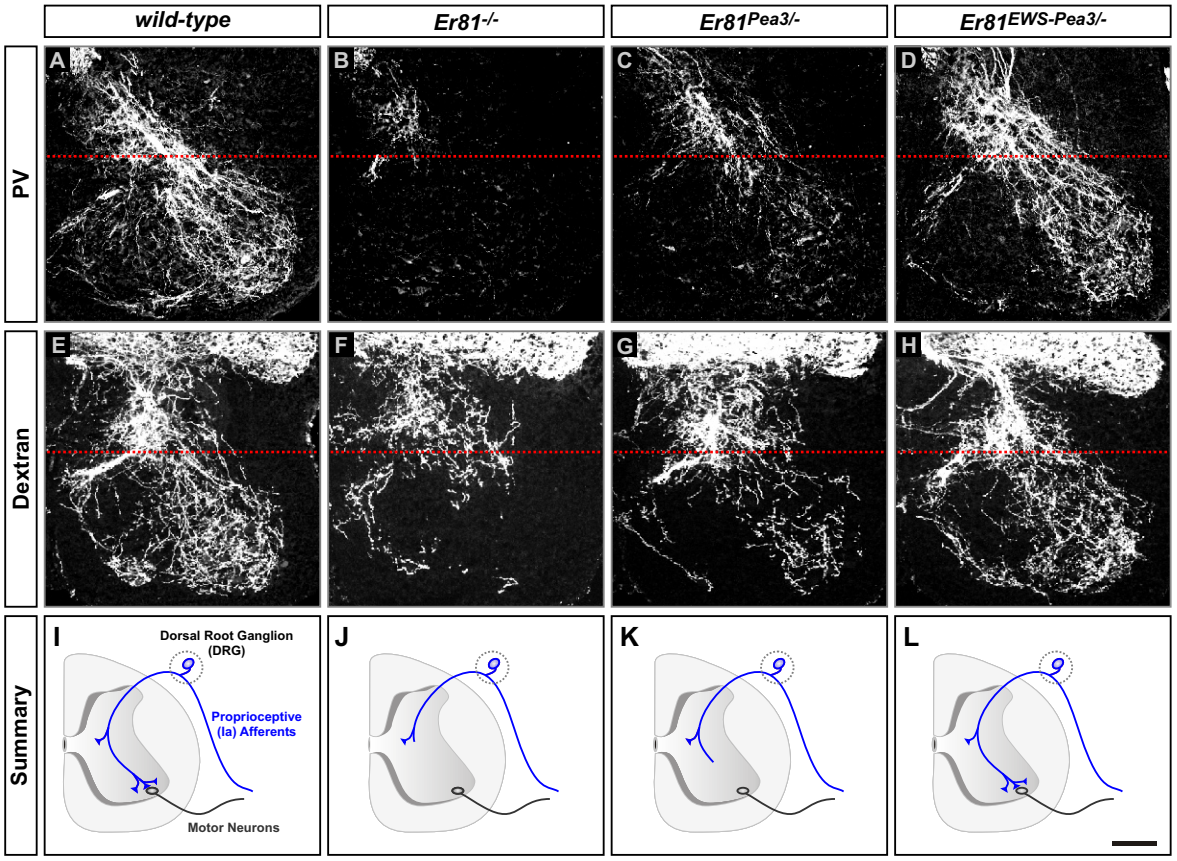
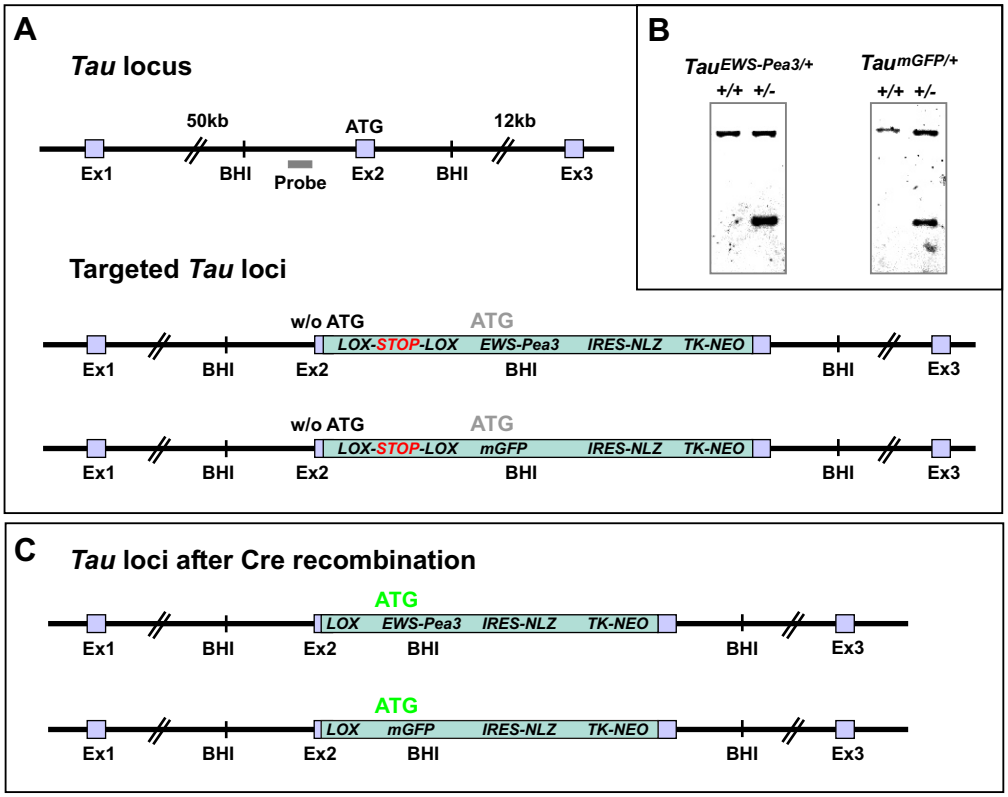


Figure 28. Rescue of Ia Proprioceptive Afferent Projections into the Ventral Spinal Cord in *Er81$^{EWS-Pea3}$* but not *Er81^{Pea3}* Mutants. (A-H) Morphological analysis of central projections at lumbar level L3 of PV^{+} DRG neurons (A-D) or all DRG sensory afferents after application of fluorescently labeled dextran to individual dorsal roots (E-H) in P0.5 (A-D) or P5 (E-H) wild-type (A, E), *Er81$^{-/-}$* (B, F), *Er81$^{Pea3/-}$* (C, G), *Er81$^{EWS-Pea3/-}$* (D, H) mice. (I-L) Schematic summary diagrams of the morphological rescue of Ia proprioceptive afferent projections (blue) into the ventral spinal cord observed in wild-type (I), *Er81$^{-/-}$* (J), *Er81$^{Pea3/-}$* (K), *Er81$^{EWS-Pea3/-}$* (L) mice. DRG are illustrated as dotted line, motor neurons are shown in black. Scale bar: (A-D) = 150 m; (E-H) = 160 m.

Since Er81 is required for the development of Ia proprioceptive projections into the ventral spinal cord, we sought to test whether the highly homologous ETS transcription factor *Pea3* or the stronger transactivating variant *EWS-Pea3* could substitute for Er81 function *in vivo*. Similar to a previously used targeting strategy (Arber et al., 2000), we introduced the coding sequence of mouse *Pea3* or *EWS-Pea3* into the *Er81* genomic locus in frame with the ATG present in the second exon by homologous recombination in embryonic stem cells (Figure 27B, C). Expression of Er81 in DRG of embryos containing integration of either *Pea3* ($Er81^{Pea3/-}$) or *EWS-Pea3* ($Er81^{EWS-Pea3/-}$) in the *Er81* locus was abolished as previously shown for $Er81^{-/-}$ mutants (Figure 27D-G; Arber et al., 2000). Expression of the calcium binding protein Parvalbumin (PV) in proprioceptive afferents of *Er81* mutant mice has been shown to be decreased ~5- to 10-fold when compared to wild-type levels (Arber et al., 2000; Figure 27H, I). A similar decrease in PV expression was detected in E16.5 proprioceptive afferents of $Er81^{Pea3/-}$ mice (Figure 27I, J). In $Er81^{EWS-Pea3/-}$ mice, however, the expression of PV in proprioceptive afferents was comparable to wild-type levels (Figure 27H, K). In addition, the number of proprioceptive afferent cell bodies within the DRG of L1 to L5 was not changed in $Er81^{EWS-Pea3/-}$ mice when compared to wild-type (data not shown).

To determine the extent of rescue of Ia proprioceptive afferent projections into the ventral spinal cord of *Er81* mutant mice achieved by expression of *Pea3* or *EWS-Pea3* we performed two assays to trace intraspinal afferent projections. We first assessed proprioceptive afferent projections by axonal labeling of PV (Figure 28A-D). In wild-type mice, PV^+ fibers invaded the ventral horn of the spinal cord, branching extensively in the proximity of motor neuron dendrites and cell bodies whereas proprioceptive afferents in $Er81^{-/-}$ mutants terminated prematurely in the intermediate region of the spinal cord (Figure 28A, B; Arber et al., 2000). $Er81^{Pea3/-}$ mice displayed projection defects similar to $Er81^{-/-}$ mutants although more proprioceptive afferent projections crossed the intermediate spinal cord to project into the ventral spinal cord (Figure 28C). In contrast, proprioceptive afferents in $Er81^{EWS-Pea3/-}$ mice invaded the ventral horn of the spinal cord extensively (Figure 28D).

To determine the extent of afferent projections into the ventral spinal cord independent of the level of PV expression in DRG neurons, we used anterograde labeling of afferent fibers by applying fluorescently labeled dextran to cut dorsal roots (Figure 28E-H). Similar to our findings using the antibody to PV, we only found extensive rescue of the projections into the ventral horn of the spinal cord in $Er81^{EWS-Pea3/-}$ mice (Figure 28H, L). In contrast, $Er81^{Pea3/-}$ mice showed only minimal increase in the amount of afferent ingrowth into the ventral horn of the spinal cord (Figure 28G, K).



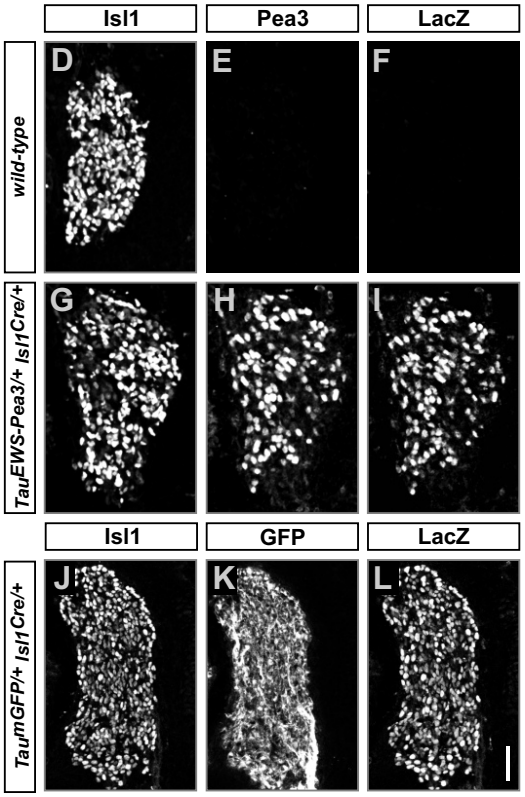


Figure 29. Generation of Mice Expressing *EWS-Pea3* or *mGFP* in Early Postmitotic DRG Neurons. (A) (Top panel) Organization of the *Tau* genomic locus in the region targeted by homologous recombination in analogy to Tucker et al., 2001. Exons 1-3 are shown as light blue boxes and the *Tau* start codon in exon 2 is indicated as ATG. The probe used to detect homologous recombination is shown as a grey box. (Middle; bottom panels) *Tau* locus after homologous recombination integrating targeting cassettes (green) into exon 2 with coincident elimination the endogenous *Tau* start codon. The integrated targeting cassettes allow for conditional expression of *EWS-Pea3* (middle) or membrane linked *eGFP* (*mGFP*; bottom) and *NLS-LacZ* (*NLZ*) upon Cre recombinase-mediated activation. In the absence of Cre recombinase, a transcriptional stop sequence flanked by *loxP* sites inhibits expression of the respective transgenes from their start codons (ATG in grey). (B) Southern blot analysis of $Tau^{EWS\text{-}Pea3/+}$ and $Tau^{mGFP/+}$ genomic DNA to detect the mutant allele (+/-). (C) In the presence of Cre recombinase, the transcriptional stop sequence in the cassettes integrated into the *Tau* locus is removed. Expression of *EWS-Pea3* (top) or *mGFP* (bottom) and *NLS-LacZ* (*NLZ*) can now occur in neurons coincidently expressing Cre recombinase and *Tau* (indicated as ATG in green). (D-L) Expression of Isl1 (D, G, J), EWS-Pea3 (E, H) or GFP (K), and LacZ (F, I, L), in E12 (D-I) or E13.5 (J-L) DRG neurons of wild-type (D, E, F), $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ (G, H, I) and $Tau^{mGFP/+}$ $Isl1^{Cre/+}$ (J, K, L) embryos. Scale bar: (D-F) = 40 m; (G-I) = 35 m; (J-K) = 50 m.

Together, these findings suggest that in the absence of *Er81*, *EWS-Pea3* but not *Pea3* can direct proprioceptive afferent projections into the ventral spinal cord (Figure 28I-L).

Premature Expression of EWS-Pea3 in Early Postmitotic DRG Neurons Leads to Axonal Projection Defects

The finding that *EWS-Pea3* can substitute for *Er81* function in proprioceptive afferents at the comparatively late developmental stage when *Er81* is normally first induced by target-derived factors, prompted us to determine whether *EWS-Pea3* can fulfill a similar function when expressed prematurely during DRG neuron differentiation, before the onset of endogenous *Er81* expression, or whether the late onset of expression is critical for this function.

To express *EWS-Pea3* in early postmitotic DRG neurons, we used a binary mouse genetic system based on Cre recombinase-mediated excision of a transcriptional stop cassette flanked by *loxP* sites. First, we generated a strain of mice conditionally expressing *EWS-Pea3* from the *Tau* locus (Figure 29A-C). Moreover, we also generated mice expressing a membrane-targeted version of enhanced green fluorescent protein (*mGFP*; De Paola et al., 2003) using the same targeting strategy (Figure 29A-C) to be able to trace axonal projections of DRG neurons that have undergone Cre-mediated recombination. Embryos positive for both $Isl1^{Cre/+}$ and $Tau^{EWS\text{-}Pea3/+}$ or $Tau^{mGFP/+}$ alleles showed efficient activation of the silent *Tau* allele in $\geq$95% of DRG neurons at all segmental levels (Figure 29D-L; data not shown).

We first assessed the influence of *EWS-Pea3* expression in early postmitotic DRG neurons on the establishment of afferent projections into the spinal cord using the $Tau^{mGFP/+}$ allele. By E13.5, wild-type GFP^+ proprioceptive afferent projections had entered the grey matter of the dorsal lumbar spinal cord close to the central canal and projected ventrally to reach the intermediate level of the spinal cord (Figure 30A). In contrast, GFP^+ sensory afferents in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos failed to invade the spinal cord and instead were found in an extreme lateral position at the dorsal root entry zone (Figure 30C). This phenotype persisted at least up to E18.5 (Figure 30B, D, data not shown) thus ruling out a developmental delay in the establishment of afferent projections in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mice. To avoid signal interference from motor neurons or interneurons expressing Isl1 for axonal tracing of central sensory afferents, we also used a mouse strain in which a fusion protein between synaptophysin and GFP is expressed under the control of the *Thy1* promoter with an expression profile restricted to DRG sensory neurons at E13.5 ($Thy\text{-}1^{spGFP}$; De Paola et al.,

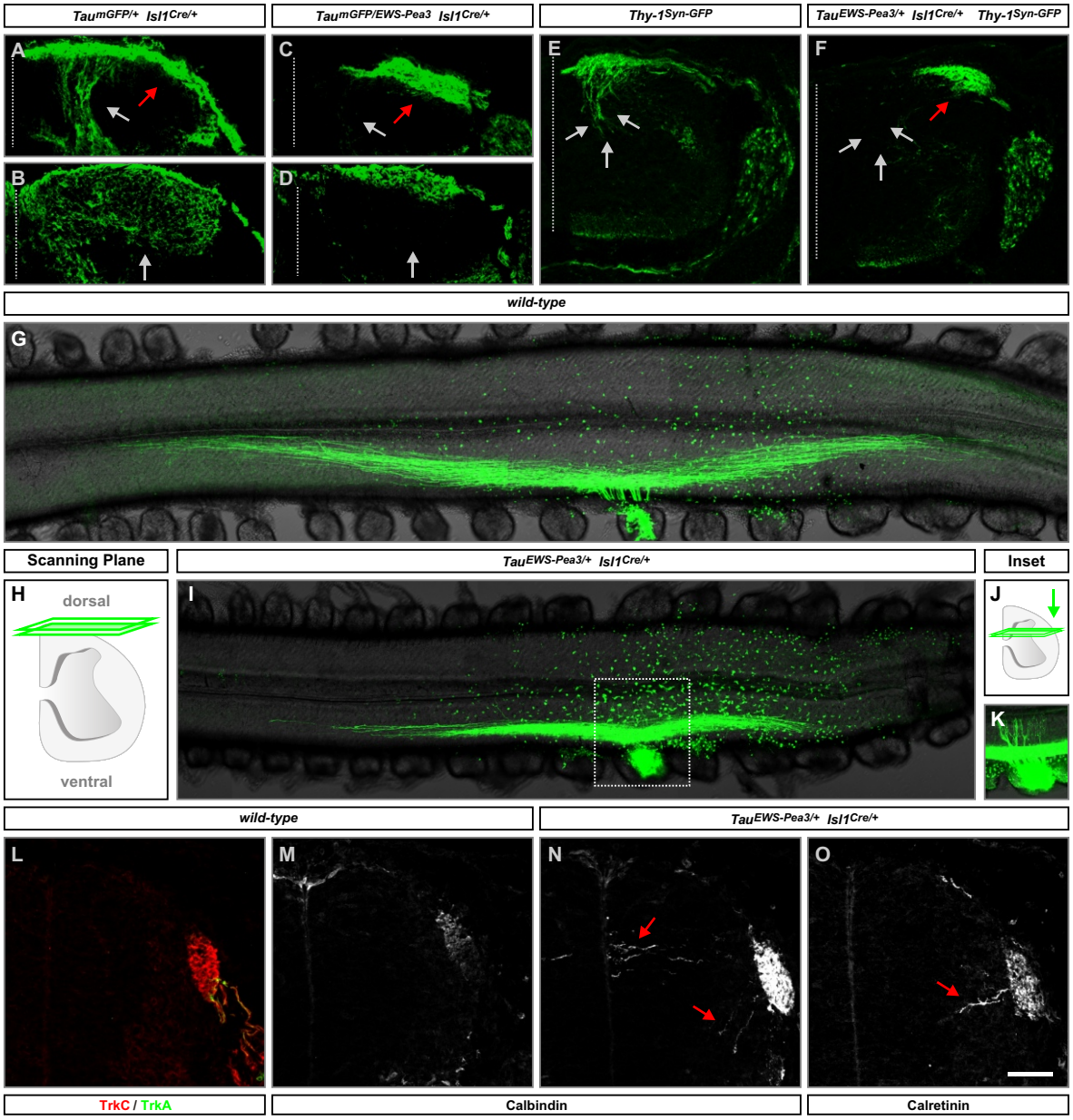


Figure 30. Defects in the Establishment of Sensory Afferent Central Projections by Expression of *EWS-Pea3* in Early Postmitotic DRG Neurons. (A-F) Visualization of sensory afferent projections (green) into the spinal cord of wild-type (A, B, E) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (C, D, F) embryos at E13.5 (A, C, E, F) and E16.5 (B, D) by Cre recombinase mediated activation of mGFP expression from the *Tau* locus (A-D) or by a *Thy-1*^{spGFP} transgene (E, F; de Paola et al., 2003). White arrows indicate normal pattern of afferent projections into the spinal cord whereas red arrows show aberrant accumulation of sensory afferents at the lateral edge of the spinal cord in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos. (G-O) Analysis of bifurcation of sensory afferent projections towards the spinal cord in wild-type (G, L, M) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (I, K, N, O) at E12 (L-O) and E13.5 (G-K). (G-K) Whole mount spinal cord of embryos after injection of fluorescently labeled dextran (green) into one DRG (lumbar level L3). Confocal scanning planes for (G, I and K) are schematically illustrated in (H and J). Inset in (I) is also shown at a deeper confocal scanning plane (J, K) to visualize aberrant axonal projections. (L-O) Transverse sections to visualize sensory afferent projections towards and/or into the spinal cord by expression of TrkA (L, green), TrkC (L, red), Calbindin (M, N), and Calretinin (N, O). Aberrant projections in (N, O) are indicated by red arrows. Scale bar: (A, C) = 60 m; (B, D) = 80 m; (E, F) = 100 m; (G, I, K) = 240 m; (L-O) = 30 m.

2003). These experiments confirmed the pronounced defect in the development of central projections in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mice (Figure 30E, F).

We next visualized the path of sensory afferent projections towards the dorsal root entry zone in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos by injecting fluorescently-labeled dextran into an individual DRG at lumbar level (L3; n=3; Figure 30G-K). Sensory afferents in E13.5 wild-type embryos bifurcated at their lateral spinal entry point, projected rostrally and caudally over ≥6 segmental levels while gradually approaching the midline (Figure 30G). Sensory afferents in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos also bifurcated at the entry point, although ~5% of afferent fibers continued to grow towards the midline (Figure 30I, K-O). While rostro-caudal projections were present in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos, afferent fibers failed to approach the midline at distal segments and continued to occupy an extreme lateral position (Figure 30I), consistent with the analysis of transverse sections.

To determine whether establishment of peripheral projections was also affected upon early *EWS-Pea3* expression, we examined the pattern of sensory innervation in skin (Figure 31) as well as the induction and innervation of muscle spindles within muscles (Figure 32). By E16.5, wild-type cutaneous sensory axons labeled by GFP expression from the *Tau* locus had established all major nerve trunks and fine axonal branches within the skin (Figure 31A, C, E). In addition, muscle spindles were innervated by GFP^+ (also $GAP43^+$) axons and expressed genes specific for muscle spindles such as *Egr3*, *Pea3* and *Erm* (Figure 32A, C, E, G, I). While sensory axons in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos reached the skin and established major nerve trunks by E16.5, only rudimentary sensory axon branching was established within the skin, a phenotype not rescued up to birth (Figure 31B, D, F; data not shown). In addition, there was a significant reduction in the number of muscle spindles (~25% of wild-type complement, n=3) as assessed by innervation and gene expression in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos (Figure 32B, D, F, H, J; data not shown).

Thus, whereas late expression of *EWS-Pea3* promoted the establishment of proprioceptive afferent projections into the ventral spinal cord, premature expression of *EWS-Pea3* in early postmitotic DRG neurons negatively interfered with establishment of projections into the spinal cord as well as to peripheral targets.

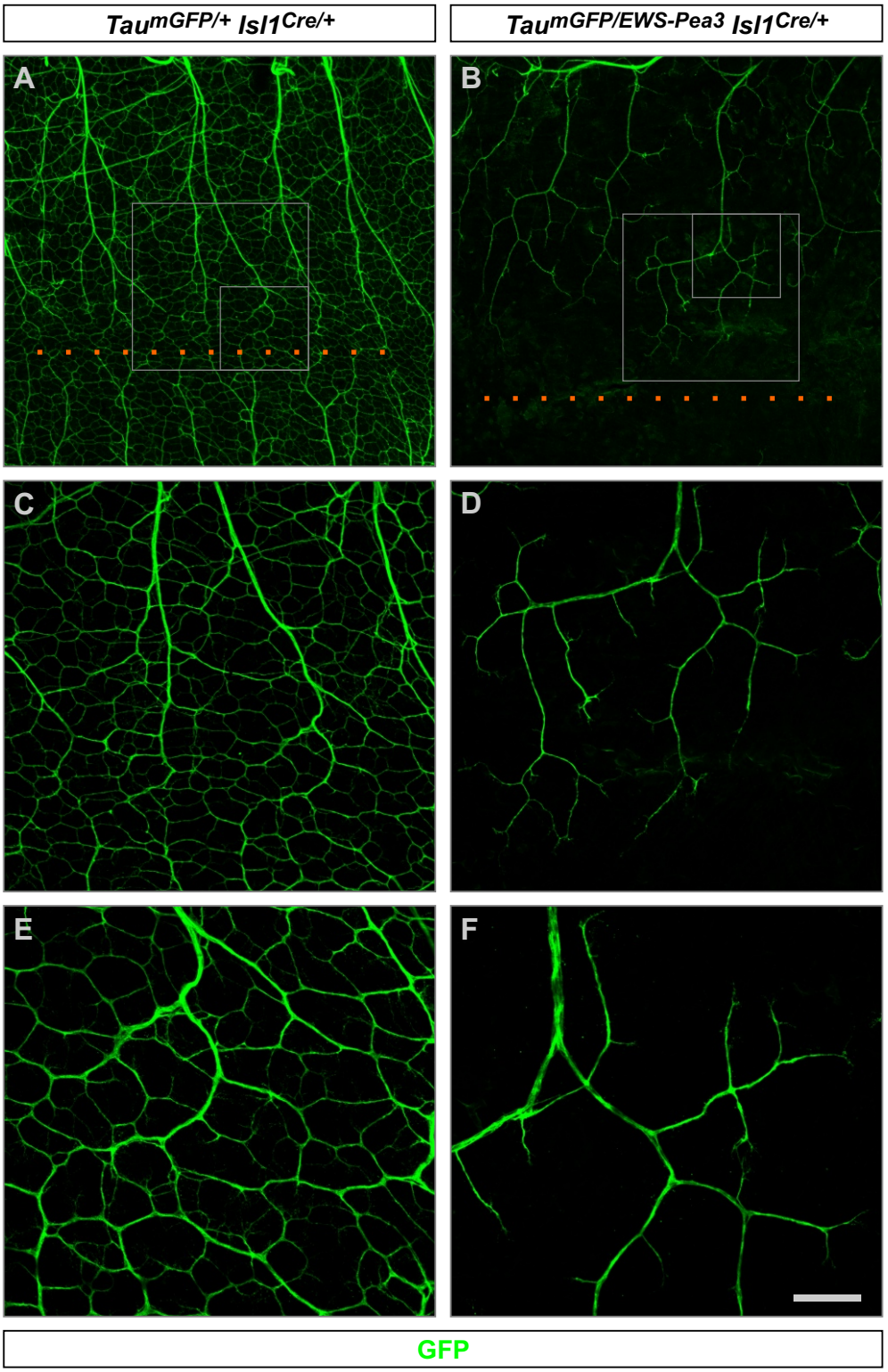


Figure 31. Defects in the Establishment of Sensory Cutaneous Afferent Peripheral Projections by Expression of *EWS-Pea3* in Early Postmitotic DRG Neurons. (A-F) Analysis of sensory afferent projections into the dorsal body wall skin of wild-type (A, C, E) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D, F) embryos at E16.5 by Cre recombinase mediated activation of mGFP (green) expression from the *Tau* locus. The midline (orange dots) is indicated. Boxes in (A, B) indicate views shown at higher magnification in (C- F). Scale bar: (A, B) = 740 m; (C, D) = 320 m; (E, F) = 160 m

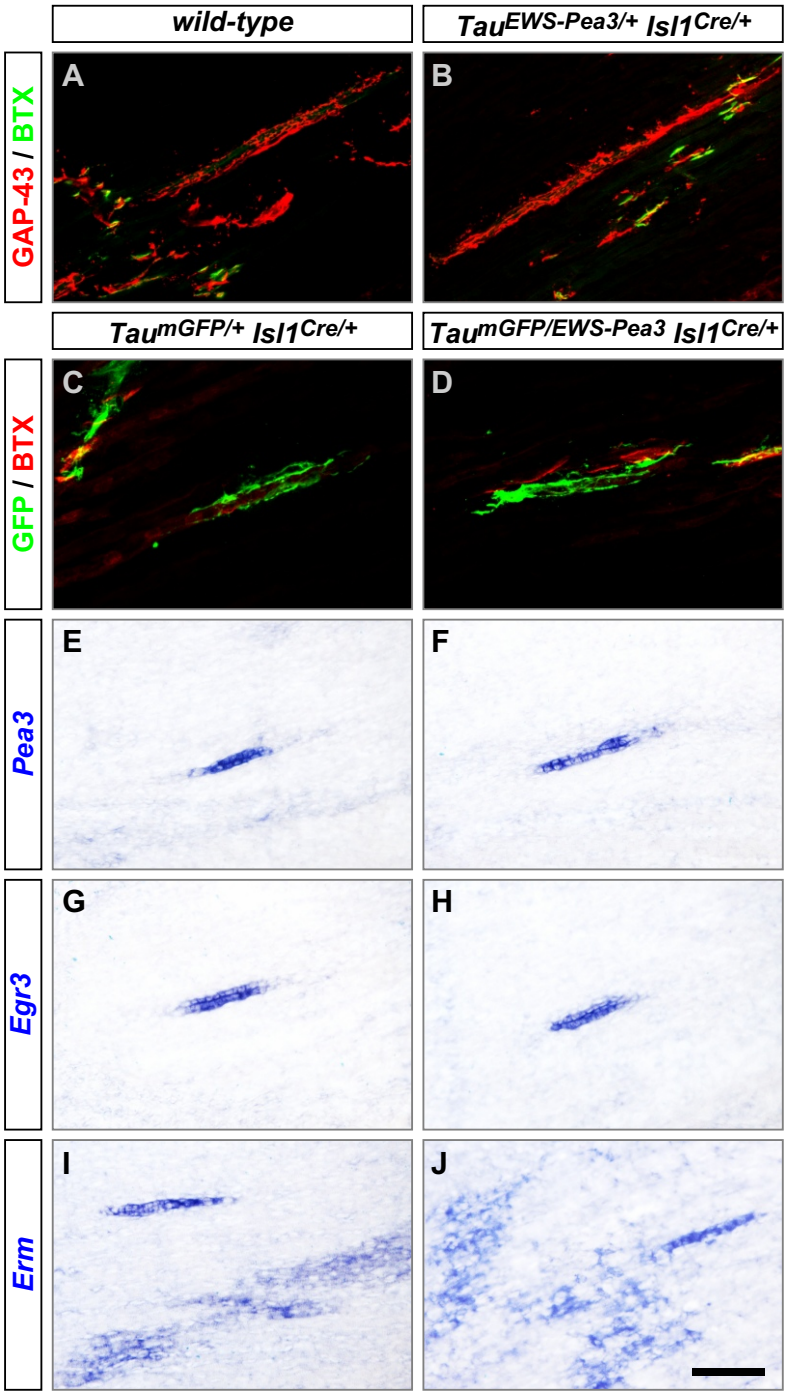


Figure 32. Muscle Spindle Differentiation in *Tau*$^{EWS\text{-}Pea3}$ Mutant Mice. (A-D) Analysis of sensory afferent projections into the muscle of wild-type (A, C) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D) embryos at E16.5 by GAP-43 expression (A, B: red) or Cre recombinase mediated activation of mGFP expression (C, D: green) from the *Tau* locus. Clusters of AChRs were revealed with -bungarotoxin (BTX; A, B: green; C, D: red). (E-J) *Pea3*, *Egr3* and *Erm* expression in intrafusal muscle fibers of wild-type (E, G, I) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (F, H, J) embryos at E16.5 using *in situ* hybridization (E, G and F, H are consecutive sections to C and D, respectively). Scale bar: (A, B) = 50 m; (C, D) = 35 m; (E-J) = 50 m.

Early Postmitotic EWS-Pea3 Expression Promotes Neurotrophin-Independent Survival and Neurite Outgrowth

To begin to address the molecular and cellular mechanisms involved in the distinct biological actions of *EWS-Pea3* at different developmental stages, we first turned to *in vitro* culture experiments. These experiments in principle permit an assessment of whether premature onset of ETS transcription factor signaling influences neuronal survival and *in vitro* neurite outgrowth of DRG neurons, two parameters prominently influenced by target-derived neurotrophic factors and their receptors.

We cultured E13.5 whole DRG explants from wild-type and $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos in the presence of NGF, NT-3 or in the absence of neurotrophins and analyzed neuronal survival and neurite outgrowth on matrigel substrate after 48 hours *in vitro*. Without neurotrophic support, very few wild-type DRG neurons survived (Figure 33A). In contrast, culturing wild-type DRG with neurotrophic factors led to neuronal survival and neurite outgrowth. Addition of NGF, which supports survival of cutaneous afferents, resulted in straight and unbranched neurite outgrowth (Figure 33B), while cultures grown in the presence of NT-3, which supports survival of proprioceptive afferents, resulted in a highly branched neurite outgrowth pattern after 48 hours *in vitro* (Figure 33C). Surprisingly, DRG neurons isolated from $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos and cultured without neurotrophic support were still alive after 48 hours *in vitro* and had established long and highly branched neurites (Figure 33D). Neither the pattern of neurite outgrowth nor neuronal survival changed significantly after application of either NGF or NT-3 (Figure 33E, F). Together, these findings suggest that early postmitotic expression of EWS-Pea3 in DRG neurons appears to uncouple survival and neurite outgrowth from a requirement for neurotrophin signaling normally observed in wild-type DRG.

To assess whether neuronal survival of DRG neurons from $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos in the absence of neurotrophic support is sufficient to explain the observed neuronal outgrowth, we analyzed DRG isolated from mice mutant in the proapoptotic gene *Bax* (White et al., 1998). As observed previously in cell culture experiments, $Bax^{-/-}$ DRG neurons survived without neurotrophic support (Lentz et al., 1999). In contrast, neurite outgrowth of $Bax^{-/-}$ DRG neurons was significantly reduced (Figure 33G) when compared to either DRG from $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos cultured in the absence of neurotrophic support (Figure 33D) or $Bax^{-/-}$ DRG neurons cultured in the presence

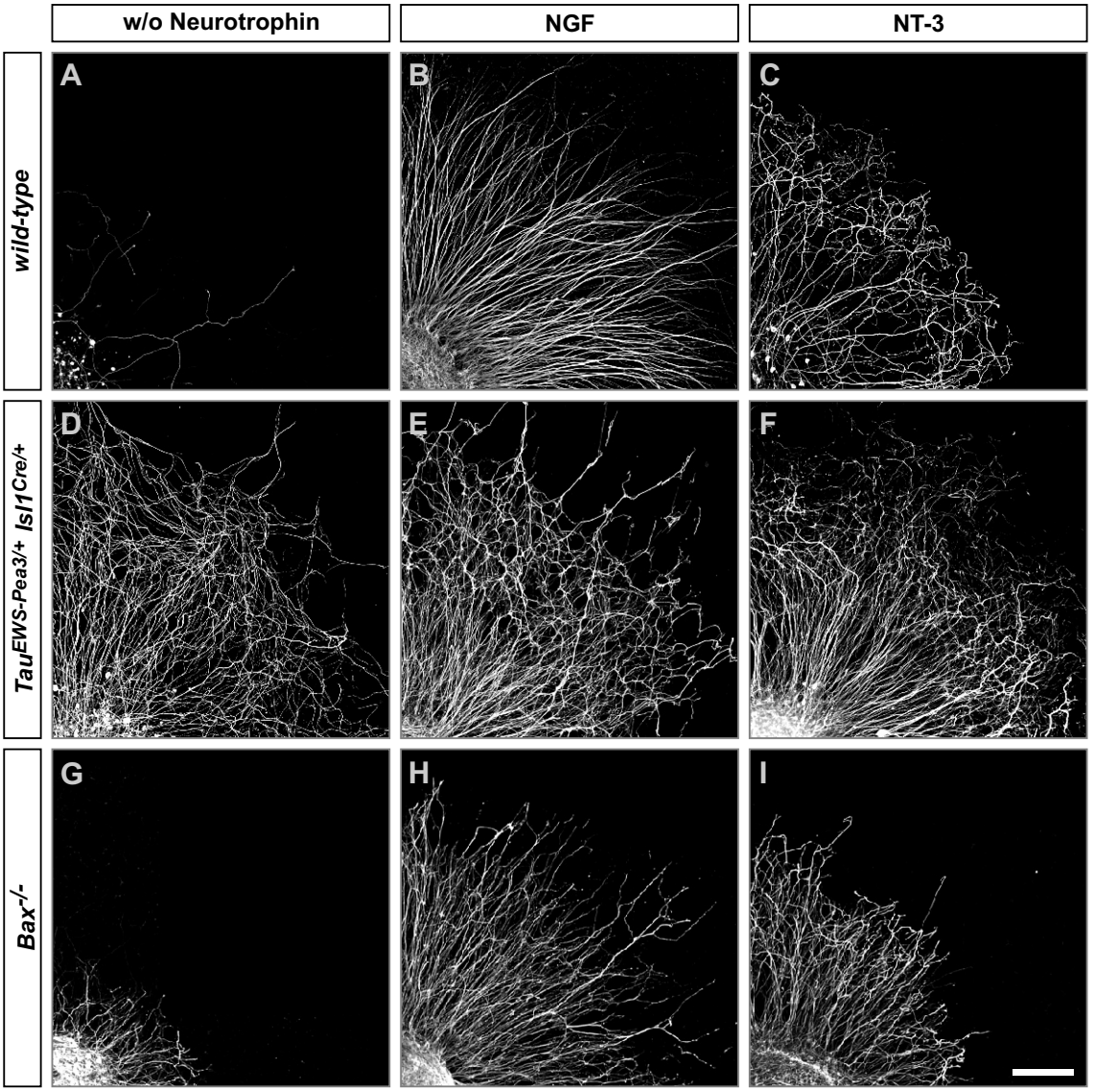


Figure 33. Neurotrophin-independent Neurite Outgrowth of *Tau*$^{EWS\text{-}Pea3}$ DRG Neurons *in vitro*. E13.5 lumbar DRG from wild-type (A, B, C), *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (D, E, F) or *Bax*$^{-/-}$ (G, H, I) embryos cultured for 48 hours without neurotrophic support (A, D, G) or in the presence of NGF (B, E, H) or NT-3 (C, F, I) were stained for expression of neurofilament to visualize axonal extensions. Scale bar: 130 m.

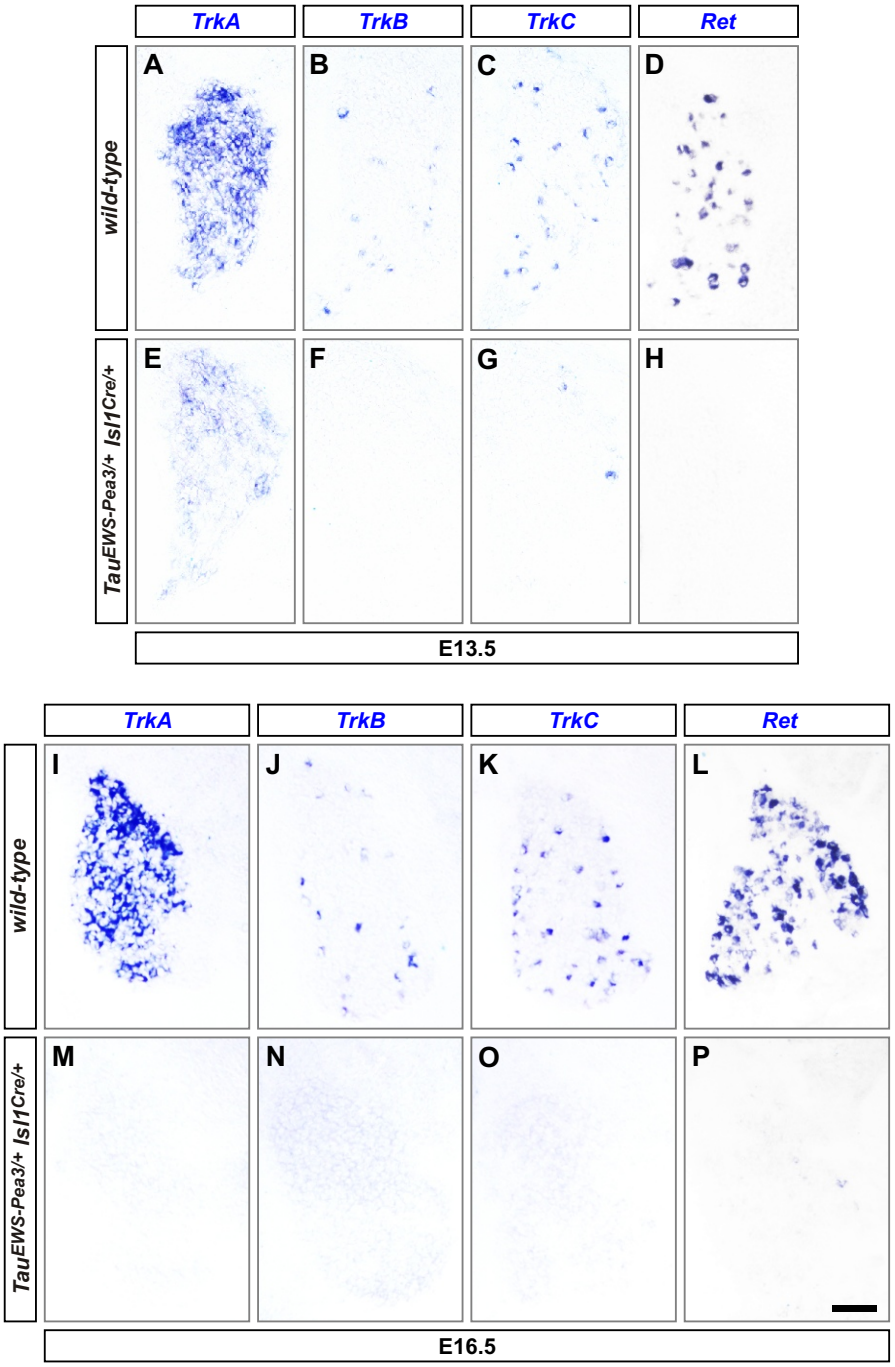


Figure 34. Loss of Trk and Ret Receptor Expression in DRG Neurons of *Tau*$^{EWS\text{-}Pea3}$ Embryos. *In situ* hybridization analysis of *TrkA* (A, E, I, M), *TrkB* (B, F, J, N), *TrkC* (C, G, K, O) and *Ret* (D, H, L, P) expression in E13.5 (A-H) and E16.5 (I-P) lumbar DRG of wild-type (A-D, I-L) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (E-H, M-P) embryos. Scale bar: (A-H) = 45 m; (I-P) = 65 m

of neurotrophic support (Figure 33H, I). Together, these findings suggest that in addition to mediating neurotrophin-independent neuronal survival, expression of *EWS-Pea3* in early postmitotic neurons also promotes neurite outgrowth in a neurotrophin-independent manner.

To begin to assess at which step of the neurotrophin signaling cascade DRG neurons from $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos have become unresponsive to the addition of neurotrophins, we assayed the expression of neurotrophin receptors in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos. Whereas expression of the neurotrophin receptors *TrkA*, *TrkB* and *TrkC* marks afferents of distinct sensory modalities in DRG of wild-type embryos (Figure 34A-C, I-K, Huang and Reichardt, 2001; Huang and Reichardt, 2003), $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos showed a severe reduction at E13.5 (Figure 34E-G) and complete absence of expression of *TrkA*, *TrkB* and *TrkC* mRNA in DRG neurons at E16.5 (Figure 34M-O). Besides, expression of *Ret*, the co-receptor for GDNF family receptors, was also absent from mutant DRG sensory neurons at E13.5 and E16.5, respectively (Figure 34D, H, L, P).

The low levels of TrkA mRNA still present in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ DRG at E13.5 shows that DRG sensory neurons initially express Trk receptors but downregulate these rapidly upon *EWS-Pea3* induction. To address this issue at higher resolution, a time course experiment of Trk protein expression was performed. Indeed, at E12 when $TrkA^+$, $TrkB^+$, and $TrkC^+$ neurons are present in wild-type DRG (Figure 35A, B), $TrkB^+$, and $TrkC^+$ cells were already significantly reduced in number but not completely absent in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant DRG (Figure 35C, D). Even more strikingly, the number of $TrkA^+$ cells was not changed significantly in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ DRG at E12 (Figure 35A, C) probably reflecting the temporal delay in the generation of cutaneous DRG sensory neurons (Ma et al., 1999). Nevertheless, the level of TrkA protein at E13.5 was very low and completely absent at E16.5 in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ (Figure 35I-L) embryos when compared to wild-type (Figure 35E-H). Furthermore, despite some TrkA protein present in E13.5 DRG in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant embryos, residual $TrkA^+$ sensory axons exhibited a severe central projection defect similar to $TrkA^-$ sensory axons as visualized with the use of the Tau^{mGFP} allele (Figure 36).

In summary, these results indicate that premature expression of EWS-Pea3 results in fast but more or less gradual downregulation of neurotrophin receptors in all classes of DRG sensory neurons although these receptors appear to be present soon after cell cycle exit.

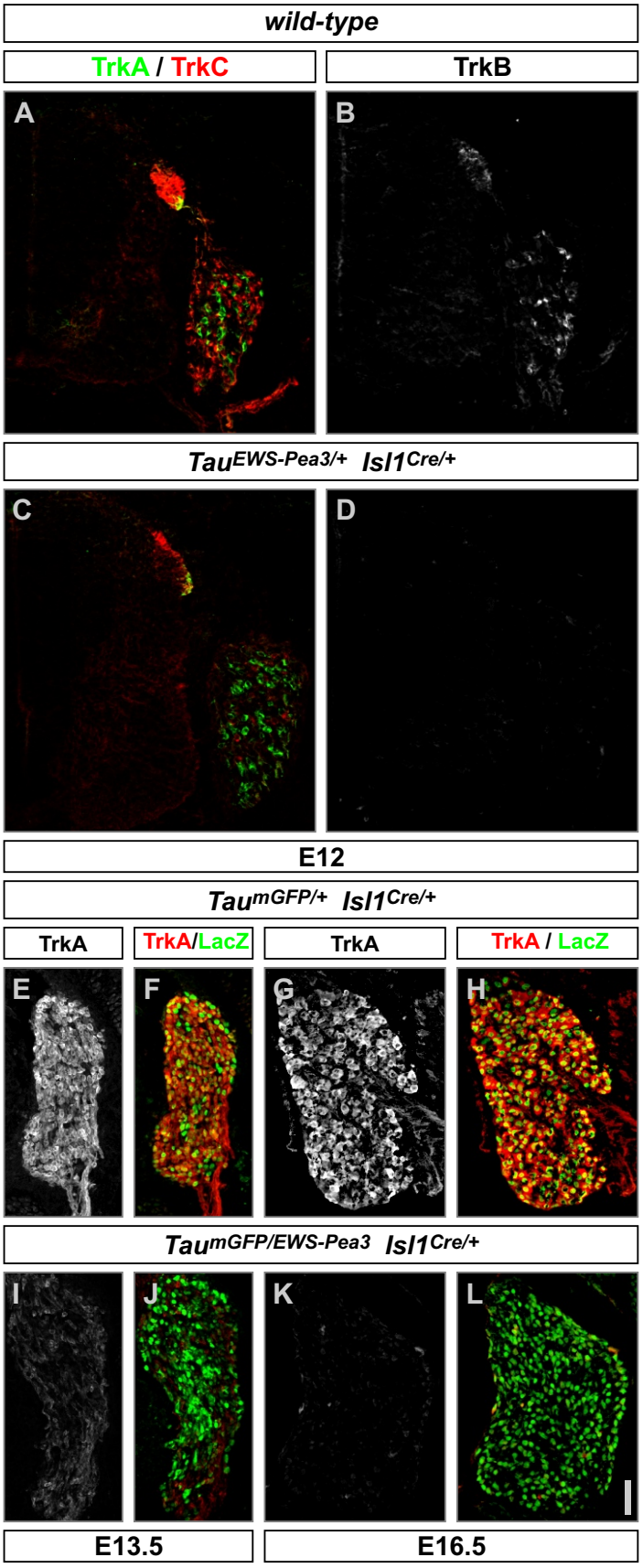


Figure 35. Gradual Loss of Trk Receptor Expression in DRG Neurons of *Tau*$^{EWS\text{-}Pea3}$ Embryos. Expression of TrkA (green A, C; white: E, G, I, K; red: F, H, J, L), TrkB (white: B, D), TrkC (red: A, C) and LacZ (green: F, H, J, L) in E12 (A-D), E13.5 (E, F, I, J) and E16.5 (G, H, K, L) lumbar DRG of wild-type (A, B), *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (C, D) *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (E-H) and *Tau*$^{mGFP/EWS\text{-}Pea3}$ *Isl1*$^{Cre/+}$ (I-L) embryos. Scale bar: (A-F, I, J) = 40 m; (G, H, K L) = 65 m

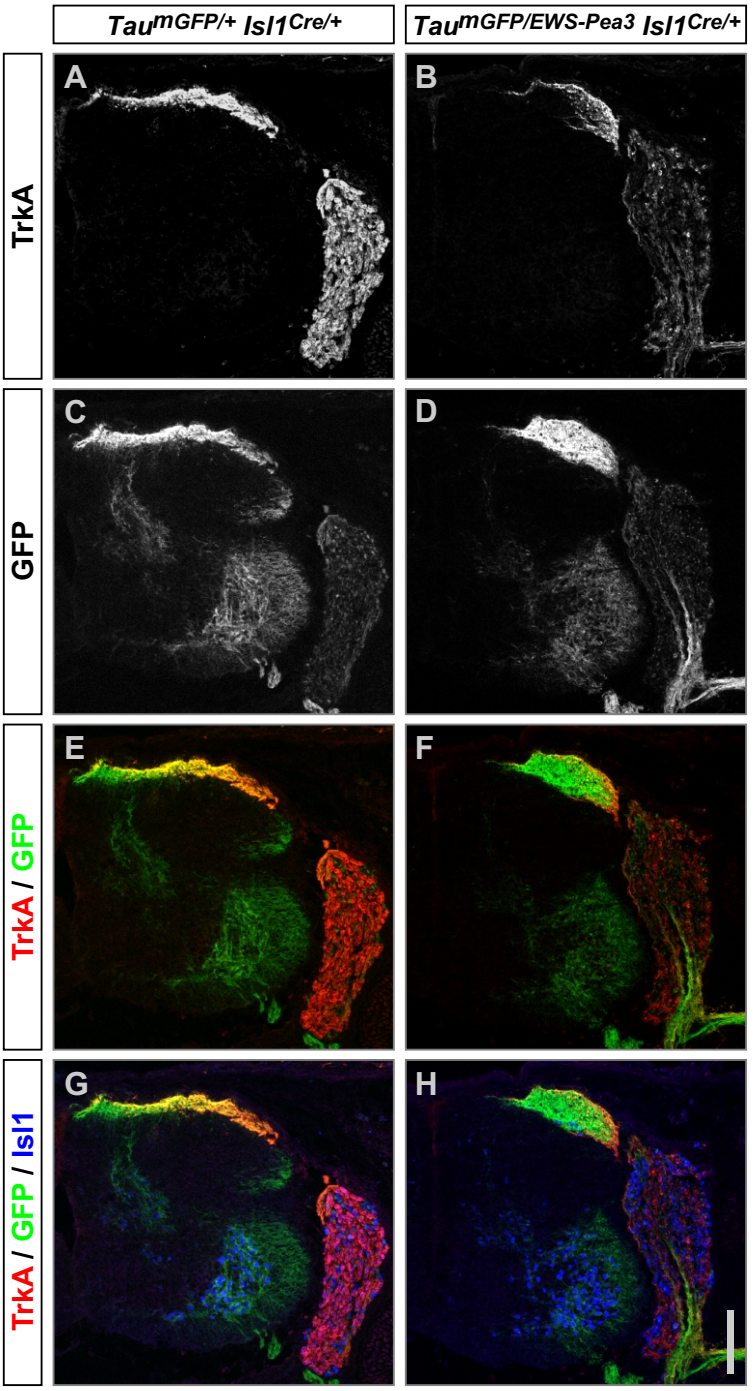


Figure 36. Central Projections of TrkA Expressing Sensory Afferents. Double-label immunohistochemistry of cutaneous sensory afferents by expression of TrkA (white: A, B; red: E-H) and all DRG sensory neurons by Cre recombinase mediated activation of mGFP (white: C, D; green: E-H) expression from the *Tau* locus in wildtype (A, C, E, G) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D, F, H) embryos at E13.5. Isl1 expressing cells are shown in (G, H: blue). Scale bar = 125 m

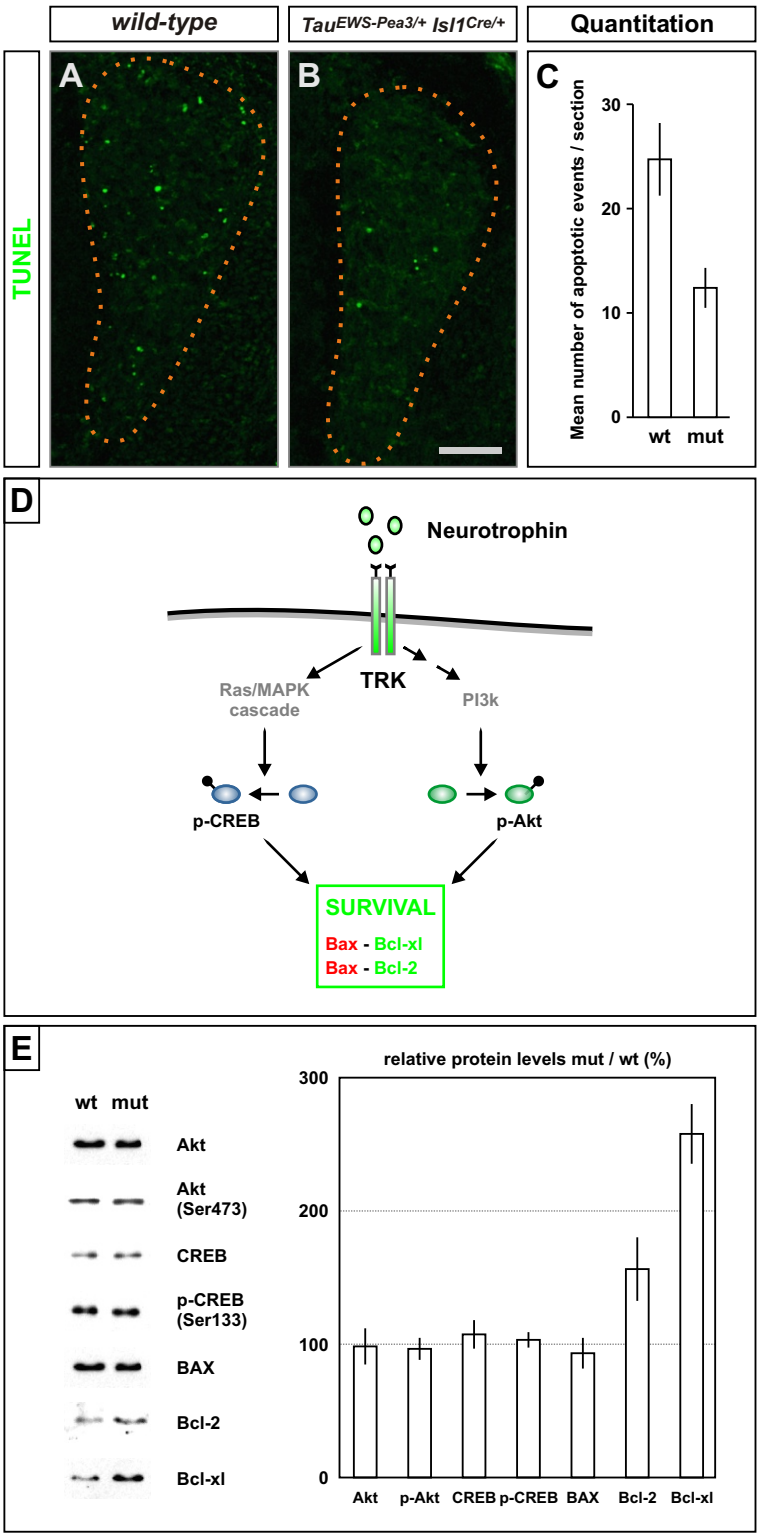


Figure 37. Increased Survival in DRG Neurons of *Tau*$^{EWS\text{-}Pea3}$ Embryos. (A-C) Analysis of neuronal cell death in E13.5 lumbar DRG of wild-type (A) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B) embryos using TUNEL (green). Quantitative analysis (n≥3 independent experiments) of apoptotic events relative to wild-type levels is shown in (C). (D) Intracellular signaling pathways downstream Trk receptors regulating apoptosis (according to Huang and Reichardt, 2003). (E) Western blot analysis of protein extracts isolated from lumbar DRG isolated from E16.5 wild-type (wt) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (mut) embryos using the following antibodies: Akt, p-Akt (Ser473), CREB, p-CREB (Ser 133), Bax, Bcl-2 and Bcl-xl. Quantitative analysis of protein levels relative to wild-type in % is shown on the right (n=3 independent experiments). Scale bar = 65 m.

Thus this gradual downregulation leading to complete absence of Trk receptor expression in DRG of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos provides a likely explanation for the lack of responsiveness of these neurons to the addition of neurotrophic factors.

We next assayed whether absence of *Trk* receptor expression in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos had an influence on naturally occurring cell death *in vivo* using TUNEL on DRG sections. We found that apoptosis was decreased by ~50% (n=3 embryos, average of ≥50 sections) in DRG of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos in comparison to wild-type (Figure 37A-C). These findings are consistent with the observation that DRG neurons from *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos in the absence of *Trk* receptors survive both *in vivo* and *in vitro*. We next analyzed whether changes in the expression of proteins known to be involved in the regulation of neuronal survival or cell death (Figure 37D) could be detected in DRG of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos. We found no significant quantitative changes in the level of Akt/p-Akt or CREB/p-CREB in DRG (Figure 37E) both of which have been shown to be key regulators of neuronal survival (Huang and Reichardt, 2003). Moreover, the level of the pro-apoptotic Bcl-2 family member Bax was not significantly reduced (Figure 37E). In contrast, the expression level of the anti-apoptotic Bcl-2 family members Bcl-xl and Bcl-2 was significantly increased when compared to wild-type levels (Bcl-2: 157%, Bcl-xl: 259%, mean average of n=3 independent experiments, Figure 37E), providing a potential molecular explanation for the enhanced neuronal survival of DRG neurons of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos in the absence of *Trk* receptor expression (Parsadanian et al., 1998; Pettmann and Henderson, 1998).

Premature ETS Signaling in DRG Neurons Interferes with Neuronal Fate Acquisition

The absence of *TrkA*, *TrkB* and *TrkC* expression in DRG neurons of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos prompted us to assess whether other genes normally expressed by subpopulations of DRG neurons are also affected in these mutants. Strikingly, both Er81 and PV, normally expressed by proprioceptive afferents in wild-type embryos (Figure 38O, P; Arber et al., 2000) were not expressed in DRG neurons of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos (Figure 38U, V). In contrast, Calretinin and Calbindin, two different calcium binding proteins also expressed by subpopulations of DRG neurons in wild-type embryos (Figure 39H, I; Zhang et al., 1990; Ichikawa et al., 1994), were induced in >95% of all DRG neurons of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos (Figure 39K, L). Since wild-type DRG neurons do not coexpress these two proteins to a large extent (Figure 40) these findings suggest that DRG neurons in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos fail to differentiate to a normal fate and instead acquire an aberrant identity distinct from any subpopulation of wild-type DRG neurons.

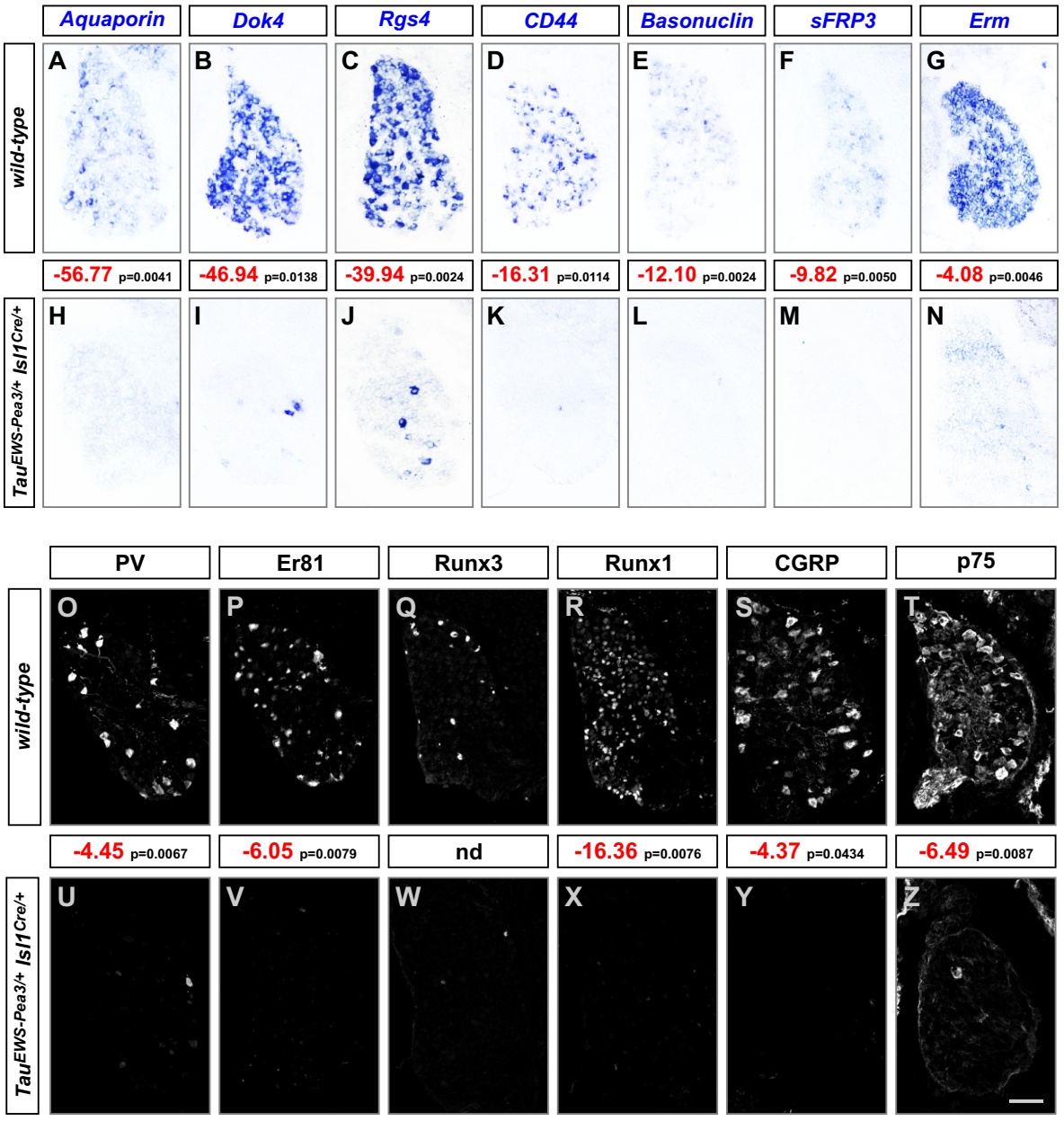


Figure 38. Gene Expression Analysis upon Induction of EWS-Pea3 in Early Postmitotic DRG Neurons - Downregulation. (A-N) *In situ* hybridization analysis of *Aquaporin* (A, H), *Dok4* (B, I), *Rgs4* (C, J), *CD44* (D, K), *Basonuclin* (E, L), *sFRP3* (F, M) and *Erm* (G, N) expression in lumbar DRG of wild-type (A-G) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (H-N) embryos at E16.5. (O-Z) Immunohistochemical analysis of PV (O, U), Er81 (P, V), Runx3 (Q, W), Runx1 (R, X), CGRP (S, Y) and p75 (T, Z) expression in E16.5 lumbar DRG of wild-type (O-T) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (U-Z) embryos. Significant changes in gene expression that were obtained by using Affymetrix gene chip technology are indicated (red numbers; p values: black; nd: not determined). Scale bar: (A-F, H-M) = 60 m; (G, N) = 70 m; (O-Z) = 50 m

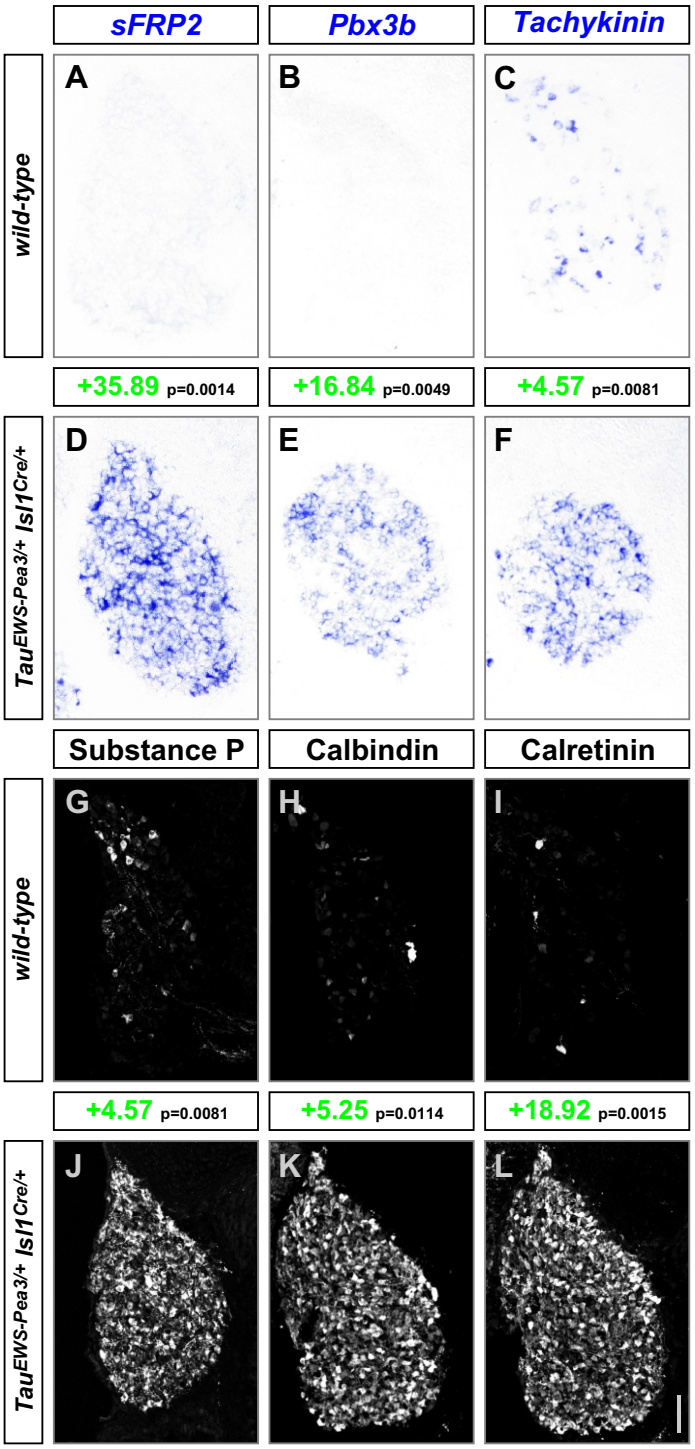


Figure 39. Gene Expression Analysis upon Induction of EWS-Pea3 in Early Postmitotic DRG Neurons - Upregulation. (A-F) *In situ* hybridization analysis of *sFRP2* (A, D), *Pbx3b* (B, E), and *Tachykinin* (C, F) expression in lumbar DRG of wild-type (A-C) and $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant (D-F) embryos at E16.5. (G-L) Immunohistochemical analysis of Substance P (G, J), Calbindin (H, K) and Calretinin (I, L) expression in E16.5 lumbar DRG of wild-type (G-I) and $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ (J-L) embryos. Significant changes in gene expression that were obtained by using Affymetrix gene chip technology are indicated (green numbers; p values: black). Scale bar: (A-F) = 60 m; (G-L) = 80 m

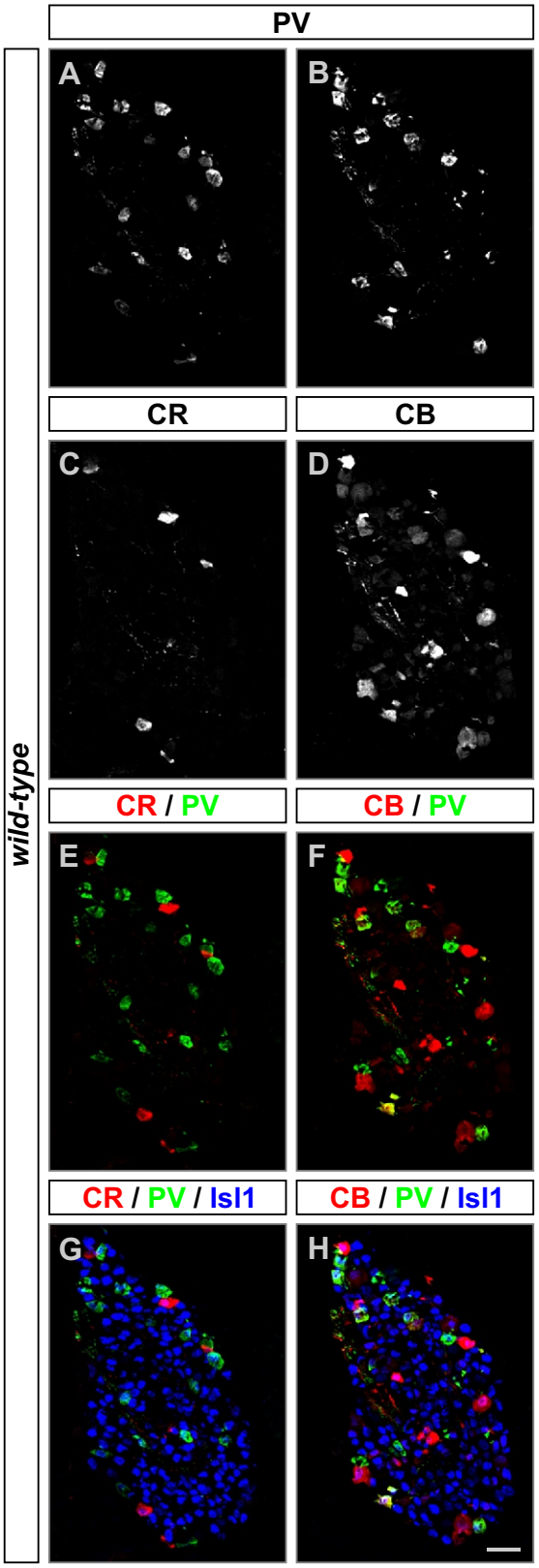


Figure 40. Calretinin and Calbindin are not Expressed in Most Proprioceptive DRG Sensory Neurons. Expression of PV (white: A, B; green: E-H), Calretinin (CR; white: C; red: E, G), Calbindin (CB; white: D; red: F, H), and Isl1 (blue: G, H) in wild-type lumbar DRG at E16.5. Scale bar: = 40 m.

The analysis with respect to cell fate acquisition of mutant *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ DRG sensory neurons was extended and the whole genomic gene expression profile was compared to the one from wild-type DRG using Affymetrix gene chip technology. Furthermore, potential candidates that were found to be differentially expressed in the mutant when compared to wild-type were analyzed using in situ hybridization and immunohistochemistry approaches to validate the results obtained *in silico* (expression data of a collection of candidate genes/proteins is shown in Figures 38, 39).

Even without going too much in detail at this point some interesting conclusions could be drawn: The vast majority of proprioceptive and cutaneous markers were downregulated in DRG sensory neurons upon premature induction of expression of *EWS-Pea3*. Nevertheless, a different genetic program appeared to be initiated in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ mutant DRG resulting in the upregulation of both subpopulation confined and ectopic DRG foreign molecules. These findings further strengthen the notion that DRG sensory neurons loose the classic proprioceptive or cutaneous cellular identity but rather acquire an aberrant cell fate.

Aberrant Cell Fate Acquisition in Tau$^{EWS\text{-}Pea3}$ *Isl1*^{Cre} *DRG Neurons Occurs in a Cell Autonomous Fashion*

Analysis of random mosaic gene expression patterns within a defined cellular subpopulation often permits to address the question whether a phenotype is based upon cell autonomous intrinsic mechanisms or caused by epigenetic means where the fate of a cell is influenced by neighboring cells. The use of mice expressing Cre recombinase from the genomic *Hb9* locus (Yang et al., 2001) generated a mosaic pattern of expression of EWS-Pea3 and LacZ within DRG in *Tau*$^{EWS\text{-}Pea3/+}$ *Hb9*$^{Cre/+}$. This mosaic expression pattern increased from rostral to caudal segmental levels in terms of the number of DRG neurons that have undergone recombination as assessed by LacZ^{+} cells at brachial and lumbar levels (Figure 41A, B). Although Hb9 is not expressed in DRG sensory neurons, expression of Cre recombinase from the genomic *Hb9* locus has occurred during early stages of neurogenesis, before neural tube closure, in a pattern of a rostro-caudal increasing gradient (with respect to the number of Hb9/Cre expressing cells) in *Hb9*$^{Cre/+}$ embryos. This early expression pattern became apparent (as a reflection of the history of expression of Hb9/Cre) at later postmitotic stages when expression of *LacZ* (and *EWS-Pea3*) was initiated from the recombined genomic *Tau* locus in *Tau*$^{EWS\text{-}Pea3/+}$ *Hb9*$^{Cre/+}$ DRG sensory neurons. Using this genetic approach it was possible to address whether premature expression of *EWS-Pea3* in DRG sensory neurons leads to downregulation of TrkA neurotrophin receptors and ectopic upregulation of Calbindin and

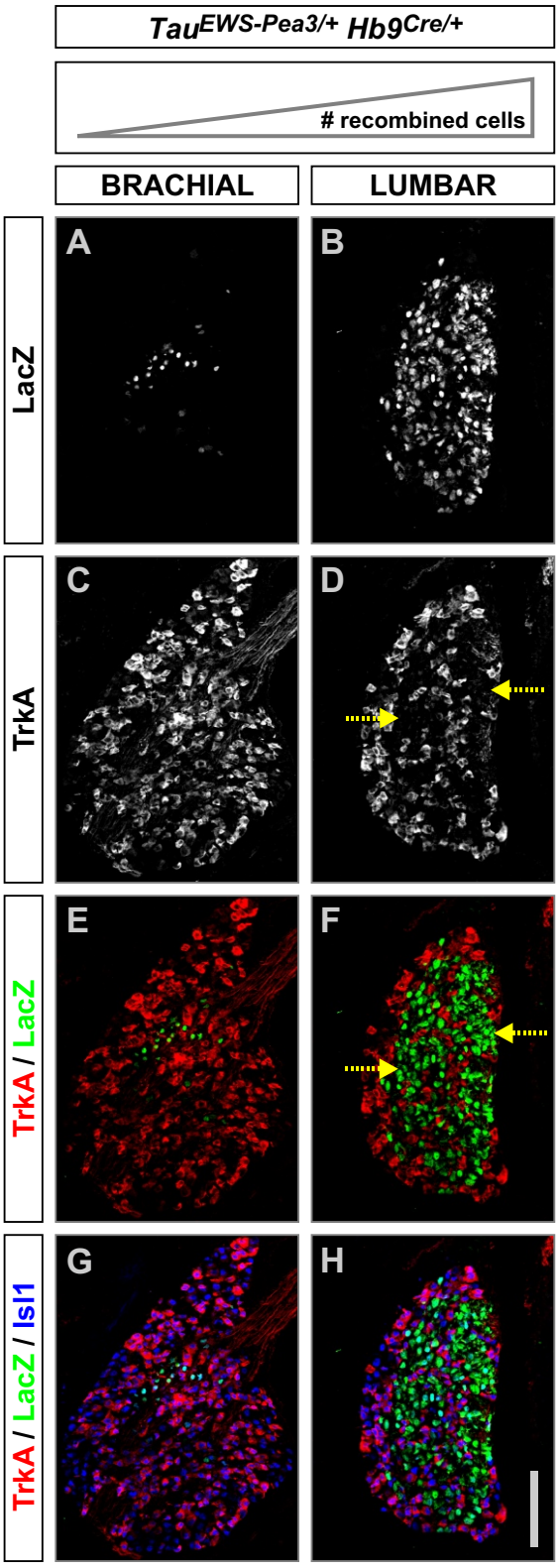


Figure 41. Loss of TrkA Expression in EWS-Pea3^{+} DRG Sensory Neurons Occurs in a Cell Autonomous Fashion. Expression of LacZ (white: A, B; green: E-H), TrkA (white: C, D; red: E-H) and Isl1 (blue: G, H) in brachial (A, C, E, G) and lumbar (B, D, F, H) DRG in *Tau*$^{EWS\text{-}Pea3/+}$ *Hb9*$^{Cre/+}$ embryos at E17.5. Scale bar = 80 m.

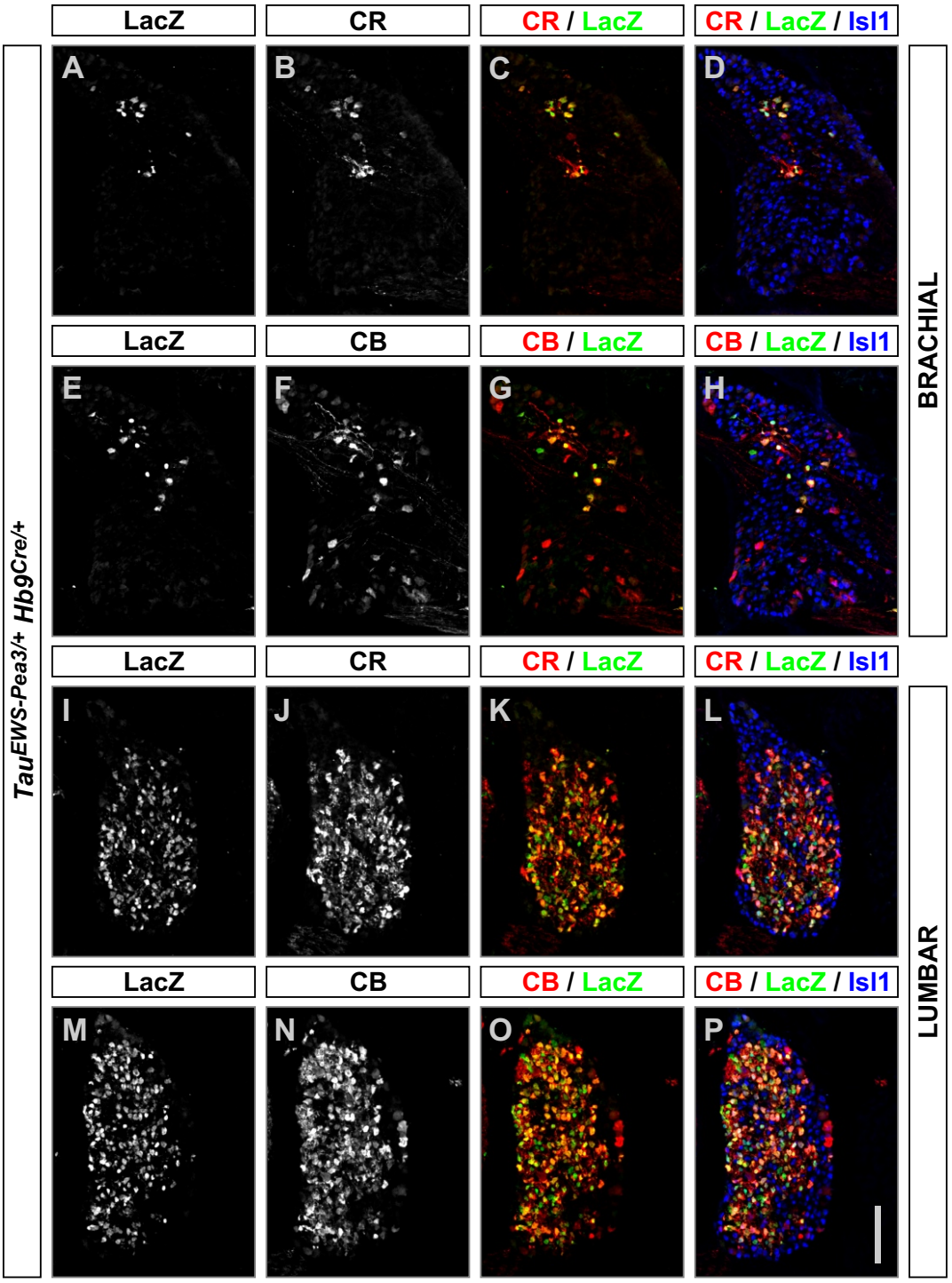


Figure 42. Ectopic Upregulation of Calretinin and Calbindin in EWS-Pea3^{+} DRG Sensory Neurons Occurs in a Cell Autonomous Fashion. Expression of LacZ (white: A, E, I, M; green: C, D, G, H, K, L, O, P), Calretinin (CR; white: B, J; red: C, D, K, L), Calbindin (CB; white: F, N; red: G, H, O, P) and Isl1 (blue: D, H, L, P) in brachial (A-H) and lumbar (I-P) DRG in *Tau$^{EWS\text{-}Pea3/+}$ Hb9$^{Cre/+}$* embryos at E17.5. Scale bar = 80 m.

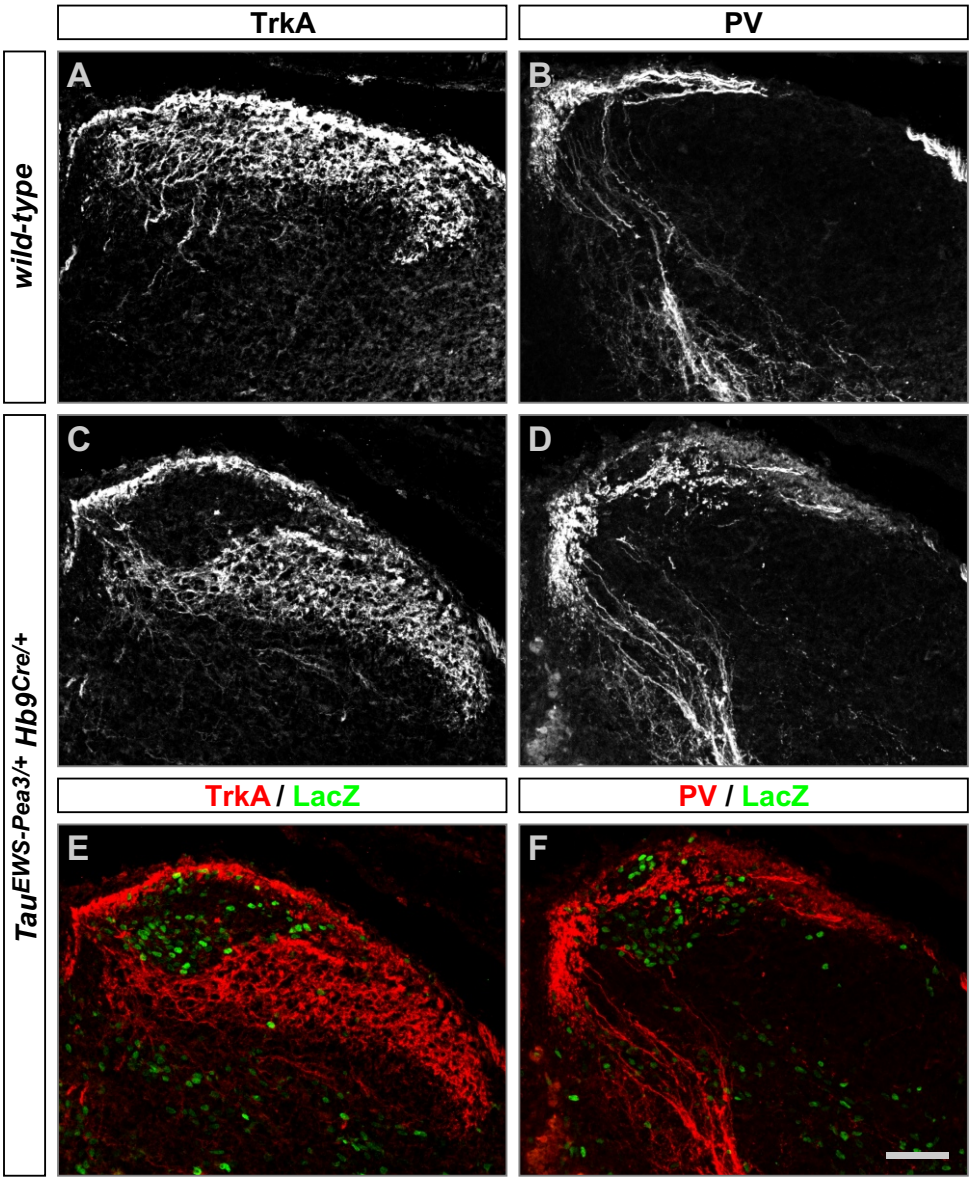


Figure 43. Central Projections of Sensory Neurons in DRG Expressing EWS-Pea3 in a Random Mosaic Pattern. Analysis of central projections of cutaneous and proprioceptive sensory neurons by expression of TrkA (white: A, C; red: E) and PV (white: B, D; red: F) at lumbar levels in wild-type (A, B) and *Tau*$^{EWS\text{-}Pea3/+}$ *Hb9*$^{Cre/+}$ mutant (C-F) embryos at E17.5. Note ectopic EWS-Pea3^{+} neurons expressing LacZ (green: E, F) in the dorsal and intermediate spinal cord. Scale bar = 80 m.

Calretinin in a cell autonomous manner. The expression of TrkA, Calbindin and Calretinin was monitored in DRG at brachial and lumbar levels at E17.5 (Figures 41, 42). Expression of TrkA in DRG of $Tau^{EWS\text{-}Pea3/+}$ $Hb9^{Cre/+}$ embryos was excluded from the cellular domains that recombined and were $LacZ^{+}$ at both brachial and lumbar levels (Figure 41C-H). Conversely, most if not all $LacZ^{+}$ cells co-expressed both Calbindin and Calretinin at rostral as well as at caudal segmental levels (Figure 42). These results provide evidence that the acquisition of an aberrant cell fate in response to premature expression of EWS-Pea3 relies upon cell autonomous transcriptional mechanisms mediated by EWS-Pea3. Interestingly, in mosaic DRG, axons of recombined EWS-$Pea3^{+}$ DRG neurons do not seem to interfere with EWS-$Pea3^{-}$ afferents establishing central projections into the spinal cord in $Tau^{EWS\text{-}Pea3/+}$ $Hb9^{Cre/+}$ embryos (Figure 43). However, wild-type PV^{+} proprioceptive and $TrkA^{+}$ cutaneous sensory axons avoided domains with ectopic $LacZ^{+}$ spinal neurons expressing EWS-Pea3 that were frequently observed as a result of recombination mediated by early Cre expression from the *Hb9* locus (Figure 43).

We next determined whether transcriptional effects of *EWS-Pea3* expression in early postmitotic neurons were restricted to DRG neurons by activating expression of *EWS-Pea3* in all somatic motor neurons as soon as they become postmitotic using $Hb9^{Cre/+}$ mice (Yang et al., 2001). We found that motor neurons in $Tau^{EWS\text{-}Pea3/+}$ $Hb9^{Cre/+}$ embryos consistently upregulated the expression of Calretinin and Calbindin when compared to wild-type embryos (data shown in detail in chapter seven, Figure 54), providing evidence for a more general action of *EWS-Pea3* in early postmitotic neurons. Together, these findings suggest that expression of EWS-Pea3 in early postmitotic neurons not only interferes with neuronal subtype specification of DRG sensory neurons but also appears to perturb differentiation in other neuronal lineages.

Late Expression of EWS-Pea3 Does not Lead to Changes in Neuronal Fate

The observed changes in cell fate acquisition and accompanying alterations in the pattern of gene expression in response to expression of *EWS-Pea3* in early postmitotic neurons led us to investigate whether similar changes are initiated by expression of *EWS-Pea3* at E13 – the normal time of onset of *Er81* – in DRG sensory neurons. Moreover, to rule out the possibility that a differential effect may be due to the different genetic strategies by which expression of EWS-Pea3 in proprioceptive afferents is achieved, we also generated a strain of mice in which *Cre* recombinase is expressed from the *Parvalbumin* (*PV*) locus (Figure 44A, B). The generation of $PV^{Cre/+}$ mice would allow us to activate expression of EWS-Pea3 at late postmitotic stages coincident with the late upregulation

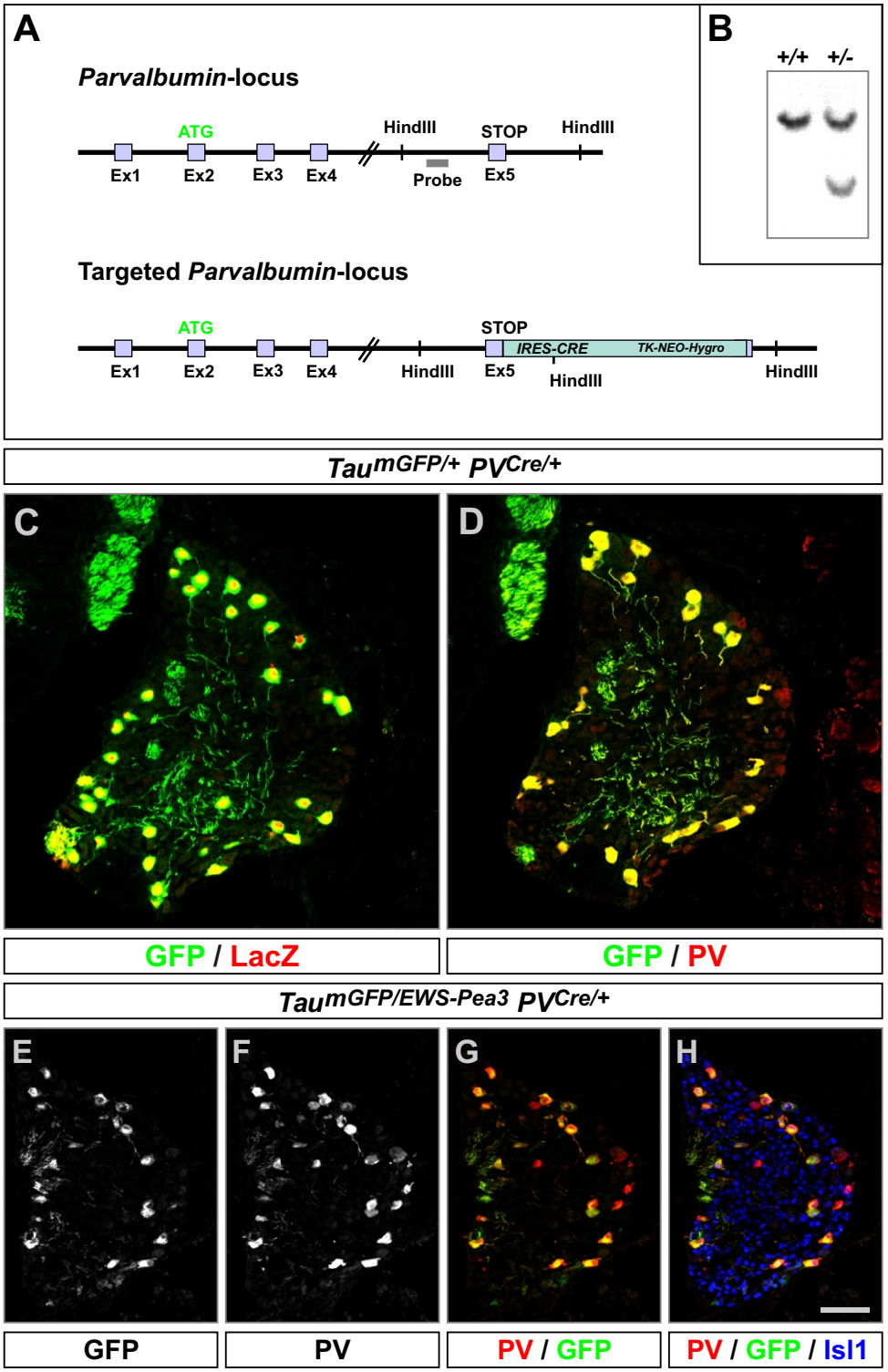


Figure 44. Generation of *PV*^{Cre} Mice. (A) (Top) Organization of the *Parvalbumin* (*PV*) genomic locus. Exons are schematically illustrated as light blue boxes, where exon 2 contains the start codon (ATG) and exon 5 contains the stop codon (STOP). Probe to screen for homologous recombination is shown as grey box. (Bottom) Schematic diagram to show the *PV* locus after the integration of an *IRES-Cre* cassette (green) 3' to the translational stop codon of *PV* using homologous recombination in ES cells. (B) Southern blot analysis of *PV*^{Cre} wild-type (+/+) and heterozygous (+/-) genomic DNA using the probe indicated in (A). (C, D) Expression of GFP (green) and LacZ (red: C) or PV (red: D) in P0 *Tau*$^{mGFP/+}$ *PV*$^{Cre/+}$ mice. Note that >90% of PV^{+} neurons coexpress GFP (D, data not shown). (E-H) Expression of GFP (white: E; green G, H), PV (white: F; red: G, H) and Isl1 (blue: H,) in lumbar DRG in *Tau*$^{mGFP/EWS-Pea3}$ *PV*$^{Cre/+}$ mutant embryos at E16.5. Scale bar: (C, D) = 30 m; (E-H) = 45 m.

of PV in DRG neurons at ~E14. To validate the use of the $PV^{Cre/+}$ allele, we followed the initiation of Cre mediated recombination in $Tau^{mGFP/+}$ $PV^{Cre/+}$ embryos. We found that expression of GFP was restricted to PV^{+} proprioceptive DRG neurons and mirrored the onset of expression of PV at ~E14.5 (Figure 44; data not shown).

We first analyzed expression of *TrkC*, a gene downregulated in DRG neurons of $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos (Figure 45A, B). In contrast, the level of expression of *TrkC* was indistinguishable from wild-type in DRG neurons of both $Er81^{EWS\text{-}Pea3/-}$ and $Tau^{EWS\text{-}Pea3/+}$ $PV^{Cre/+}$ embryos (Figure 45A, C, D). Second, we assayed expression of Calbindin and Calretinin in DRG neurons of the different mutants (Figures 45, 46, data not shown). We found that expression of Calbindin and Calretinin only coincided with expression of LacZ in DRG neurons of $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ embryos (Figures 45F; 46M, N), but that no overlap in expression in both $Er81^{EWS\text{-}Pea3/-}$ and $Tau^{EWS\text{-}Pea3/+}$ $PV^{Cre/+}$ embryos could be observed (Figures 45G, H; 46O, P; data not shown). Together, these findings demonstrate that expression of *EWS-Pea3* in the same neurons at premature or appropriately late developmental stages leads to significantly different transcriptional readouts (Figure 45I).

DISCUSSION

This study provides evidence for a target-induced developmental switch in DRG neurons to respond to ETS transcription factor signaling with a developmentally appropriate program of neuronal maturation, including target invasion and branching. We show that premature induction of ETS transcription factor signaling in lineage-committed DRG neurons results in a shift of the normal developmental program towards an aberrant cell fate, thus arguing for the necessity of target-induced and thus temporally regulated upregulation of ETS transcription factor signaling (Figure 47). More generally, our results suggest that temporally-regulated activation of transcriptional programs coupling to lineage determination programs established at early developmental stages represents an important mechanism to orchestrate late steps of neuronal maturation. Our experiments also begin to address the molecular specificity of ETS transcription factor signaling in the process of neuronal differentiation and provide genetic evidence that *Er81* function within proprioceptive afferents cannot be substituted for by the highly homologous ETS transcription factor *Pea3* but by the ETS fusion gene *EWS-Pea3*. We discuss our findings with

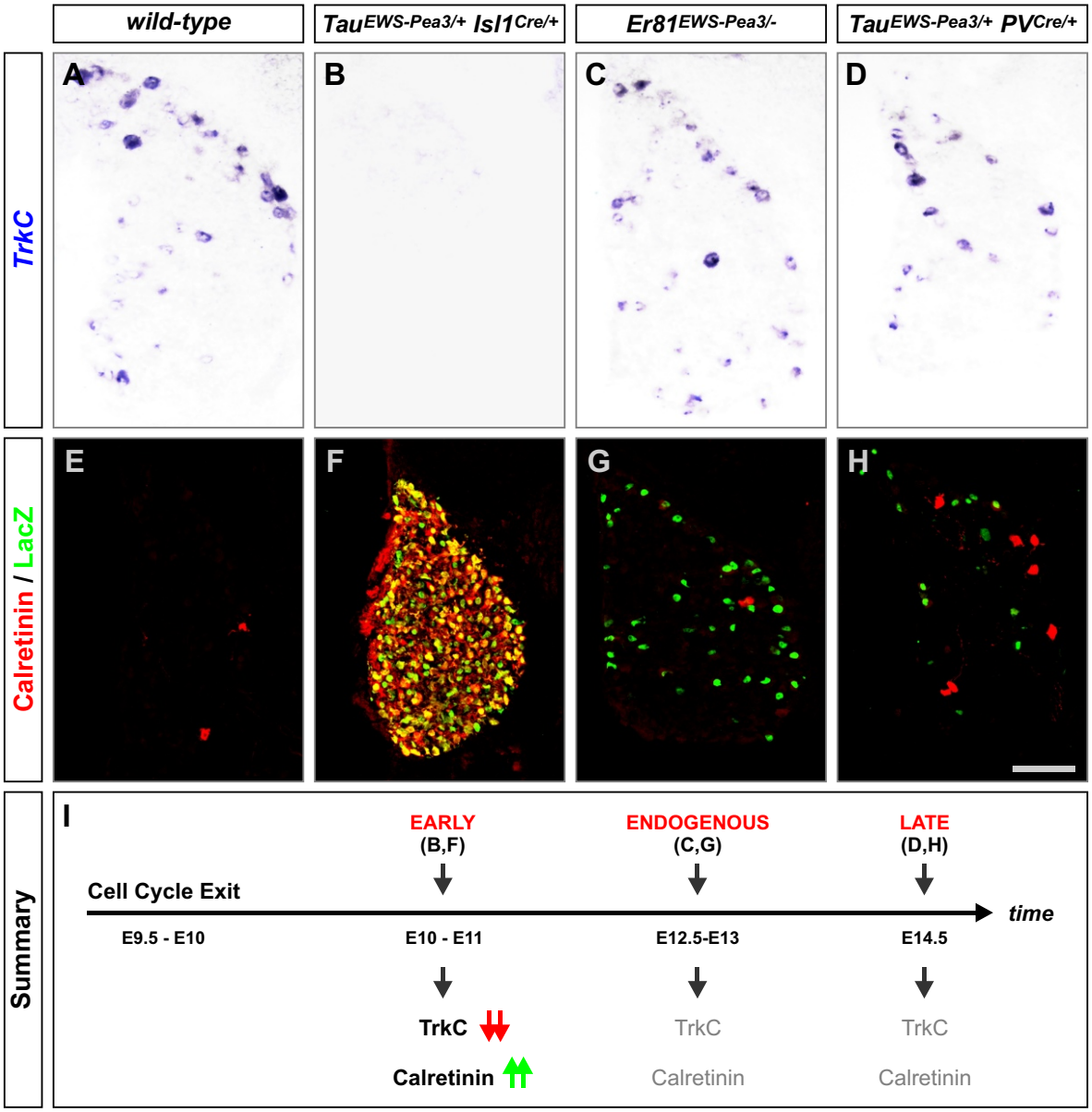


Figure 45. Gene Expression Analysis upon Induction of EWS-Pea3 in Early or Late Postmitotic DRG Neurons. (A-H) Analysis of *TrkC* expression by *in situ* hybridization (A-D) or Calretinin (red) and LacZ (green) expression by immunohistochemistry (E-H) on E16.5 lumbar DRG of wild-type (A, E), *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, F), *Er81*$^{EWS\text{-}Pea3/-}$ (C, G) and *Tau*$^{EWS\text{-}Pea3/+}$ *PV*$^{Cre/+}$ (D, H) embryos. (I) Summary diagram illustrating deregulation of TrkC (red arrows, downregulation) and Calretinin (green arrows, upregulation) expression upon early (B, F) induction of EWS-Pea3 expression in DRG neurons (B, F; E10-E11, i.e. shortly after cell cycle exit, E9.5-10). In contrast, activation of EWS-Pea3 from the endogenous *Er81* locus (C, G; E12.5-13) or via Cre recombinase expression from the *PV* locus activating expression from the *Tau* locus late (D, H; E14.5) does not interfere with the normal expression of *TrkC* and Calretinin (shown in grey). Scale bar: (A-D) = 65 m; (E-H) = 80 m.

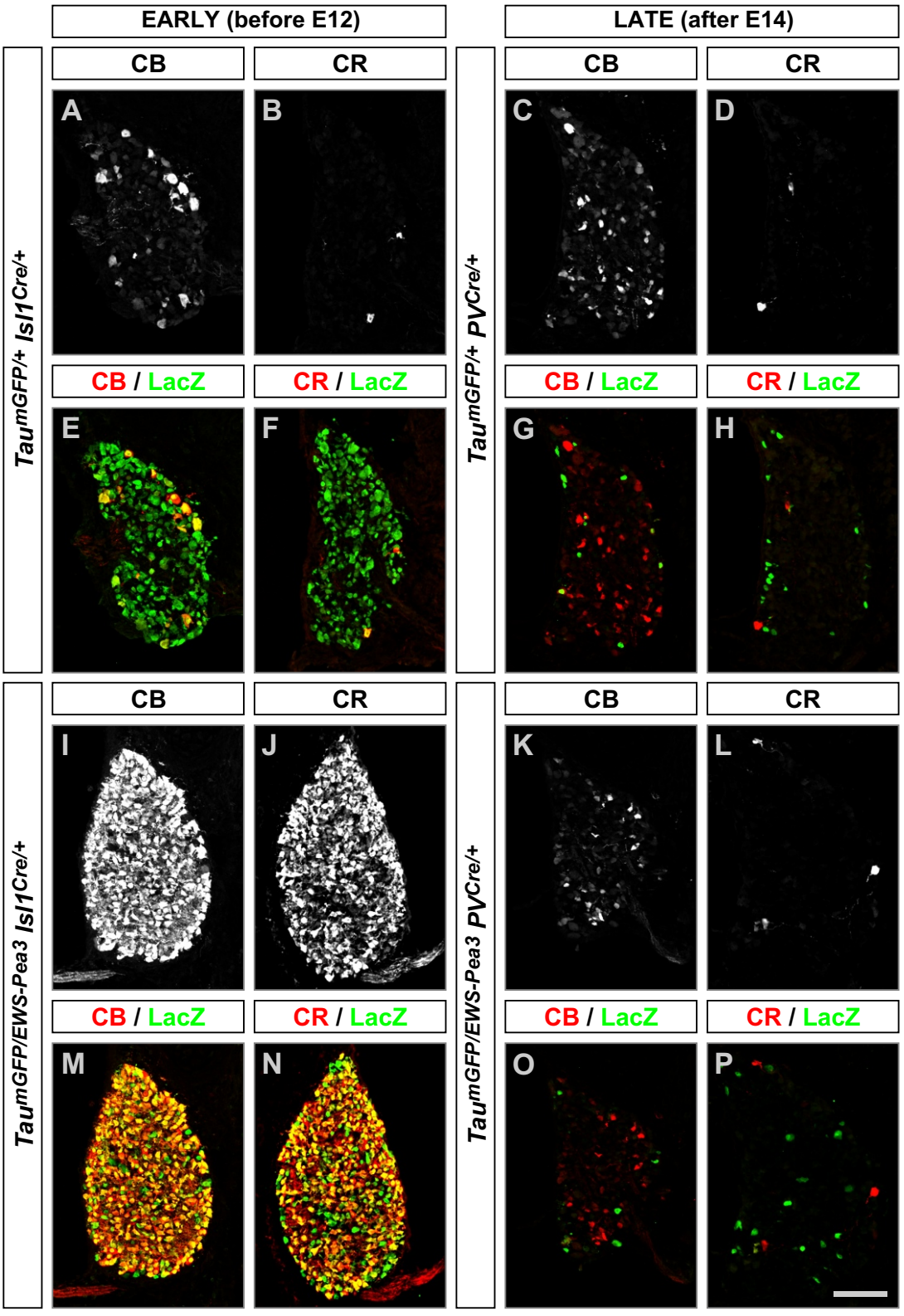


Figure 46. Expression of Calbindin and Calretinin upon Induction of EWS-Pea3 in Early or Late Postmitotic DRG Neurons. Expression of Calbindin (CB; white: A, C, I, K; red: E, G, M, O), Calretinin (CR; white: B, D, J, L; red: F, H, N, P) and LacZ (green: E-H, M-P) in lumbar DRG in *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (A , B, E, F), *Tau*$^{mGFP/+}$ *PV*$^{Cre/+}$ (C, D, G, H), *Tau*$^{mGFP/EWS\text{-}Pea3}$ *Isl1*$^{Cre/+}$ (I, J, M, N), and in *Tau*$^{mGFP/EWS\text{-}Pea3}$ *PV*$^{Cre/+}$ (K, L, O, P) embryos at E16.5. Scale bar = 70 m.

respect to the role of temporally controlled transcription factor programs in neuronal maturation and the function of ETS transcription factor specificity in the assembly of the monosynaptic reflex circuit.

Strict Temporal Requirement for Onset of ETS Transcription Factor Signaling in DRG Sensory Neurons

Progressive steps of neuronal differentiation are controlled by tightly regulated developmental programs during which successive steps in the cascade of differentiation are initiated sequentially within a specific postmitotic neuronal lineage. The differentiation of postmitotic DRG sensory neurons represents a particularly amenable system to study how transcriptional programs activated at late developmental steps intersect with prespecification of a neuronal fate initiated during early postmitotic differentiation.

Induction of Er81 expression in proprioceptive afferents is controlled by peripheral NT-3 as axons reach the vicinity of target muscles and thus occurs only approximately three days after proprioceptive neurons become postmitotic (Arber et al., 2000; Patel et al., 2003). This temporally delayed and target-induced upregulation of ETS transcription factor expression several days after a neuronal lineage of a specific identity first emerges represents a more general feature for both the expression of Er81 and Pea3 in motor neuron pools and DRG sensory neurons (Lin et al., 1998) and thus raises the question of the functional role for this temporal delay.

Our experiments now begin to address this issue by comparing the consequences of expression of the ETS transcription factor variant *EWS-Pea3* in early postmitotic DRG neurons and in DRG neurons at the time of normal onset of *Er81* expression. We found major differences in the establishment of axonal projections upon differential temporal expression of *EWS-Pea3* in proprioceptive afferent neurons. Expression of *EWS-Pea3* at the developmentally appropriate time was capable of rescuing the defect in proprioceptive afferent projections into the ventral spinal cord in *Er81* mutant mice, thus substituting for Er81 to control target invasion. In contrast, expression of *EWS-Pea3* in DRG neurons at premature developmental stages severely interfered with establishment of both peripheral and central projections of DRG neurons. Er81 and Pea3 are key mediators in proprioceptive afferents and motor neurons respectively, to control the developmental transition from long-distance axonal outgrowth to terminal branching and target invasion (Arber et al., 2000, Livet et al., 2002). Indeed, we found that DRG neurons from embryos expressing EWS-

Pea3 at early postmitotic stages were capable of axon outgrowth and extensive branching in two-dimensional cultures on matrigel in the absence of neurotrophins *in vitro*. Interestingly, three-dimensional culture experiments in collagen gel failed to result in axon outgrowth from the explants despite neuronal survival (Hippenmeyer and Arber, unpublished observation). From these findings, it is tempting to speculate that premature induction of a branching program in DRG neurons interferes with long-distance axon outgrowth thus resulting in lack of target innervation *in vivo* or lack of outgrowth in collagen gel *in vitro*. Together, our current observations provide evidence that premature ETS transcription factor signaling interferes with the normal developmental shift from axon outgrowth to target invasion and thus does not allow appropriate neuronal differentiation to progress.

Differences in the establishment of axonal projections were paralleled by differential gene expression upon presence of *EWS-Pea3* in early or late postmitotic proprioceptive DRG neurons. Expression of *EWS-Pea3* in lineage-committed postmitotic DRG neurons resulted in rapid downregulation of *Trk* receptor expression in DRG neurons as well as absence of expression of other genes expressed selectively by subpopulations of DRG neurons. Strikingly, it also resulted in ectopic upregulation of *Calbindin* and *Calretinin*, normally not expressed by proprioceptive DRG neurons. In contrast, expression of *EWS-Pea3* after the target-induced switch from long distance outgrowth to target invasion has occurred did not result in upregulation of *Calbindin* and Calretinin neither did it influence expression of *TrkC* in proprioceptive afferents. Thus, downstream gene expression changes in response to EWS-Pea3 expression in early postmitotic neurons appear to be quite distinct from those in more mature DRG neurons.

While we cannot provide direct evidence that the downstream gene expression changes we observed are directly mediated by expression of *EWS-Pea3* in early postmitotic neurons, we found that upregulation of *Calbindin* and *Calretinin* was not only observed in DRG neurons but could also be detected in motor neurons of the spinal cord (see chapter seven for detailed analysis and discussion). These findings suggest that the action of *EWS-Pea3* may not solely be restricted to early postmitotic DRG neurons but may act more generally in other neuronal contexts. Within postmitotic DRG neurons, however, the action of *EWS-Pea3* becomes progressively shifted with time, such that at a stage when *Er81* is normally first expressed, direct or indirect downstream genes normally controlled by Er81 are now preferentially activated by EWS-Pea3 thus leading to anatomical rescue of proprioceptive afferent projections into the ventral horn of the spinal cord of *Er81* mutant mice.

EWS-Pea3 Supports Neurotrophin-Independent Neurite Outgrowth and Survival

One striking observation of this study is that expression of EWS-Pea3 at premature developmental stages promotes neuronal survival without a requirement for neurotrophic support and in complete absence of Trk receptor expression. Upregulation of the anti-apoptotic Bcl-2 family members Bcl-xl and Bcl-2 upon early postmitotic expression of EWS-Pea3 in DRG neurons could be a direct transcriptional consequence since expression of both genes has been shown to be regulated by ETS transcription factors in other cellular contexts and both promoters have been shown to contain multiple ETS DNA binding elements (Lesault et al., 2002; Irvin et al., 2003). Together, these findings could thus provide a potential molecular explanation for the reduction in apoptotic cell death in DRG neurons observed in our study (Martinou et al., 1994; Parsadanian et al., 1998; Pettmann and Henderson; 1998). After the initial phase of neurotrophin-dependence, not all DRG sensory neurons require the continued presence of neurotrophins for survival. Indeed, some DRG neurons shift their neurotrophic dependence at late steps of neuronal differentiation (Bennett et al., 1996; Molliver and Snider, 1997; Molliver et al., 1997) and adult DRG sensory neurons can be kept in culture without the addition of neurotrophic support (Lindsay and Harmar, 1989). It is tempting to speculate that one feature of premature EWS-Pea3 expression in DRG sensory neurons is to uncouple neuronal survival and neurite outgrowth from the requirement for neurotrophic support at a premature stage of neuronal differentiation.

Our findings are similar but distinct from experiments in which neuronal apoptosis in neurotrophin or Trk receptor mutants is circumvented by coordinate elimination of the proapoptotic gene Bax (Patel et al., 2000; Patel et al., 2003). Elimination of TrkA receptor signaling perturbs establishment of peripheral projections of cutaneous afferents whereas establishment of central projections does not appear to be affected (Patel et al., 2000). In the absence of NT-3 signaling, development of central as well as peripheral proprioceptive afferent projections is perturbed (Patel et al., 2003). In contrast, we found more pronounced defects in the establishment of central than peripheral projections for all DRG neurons upon *EWS-Pea3* expression at premature stages of DRG neuron differentiation. Thus the absence of Trk receptor expression seems to be only partially responsible for the observed phenotype.

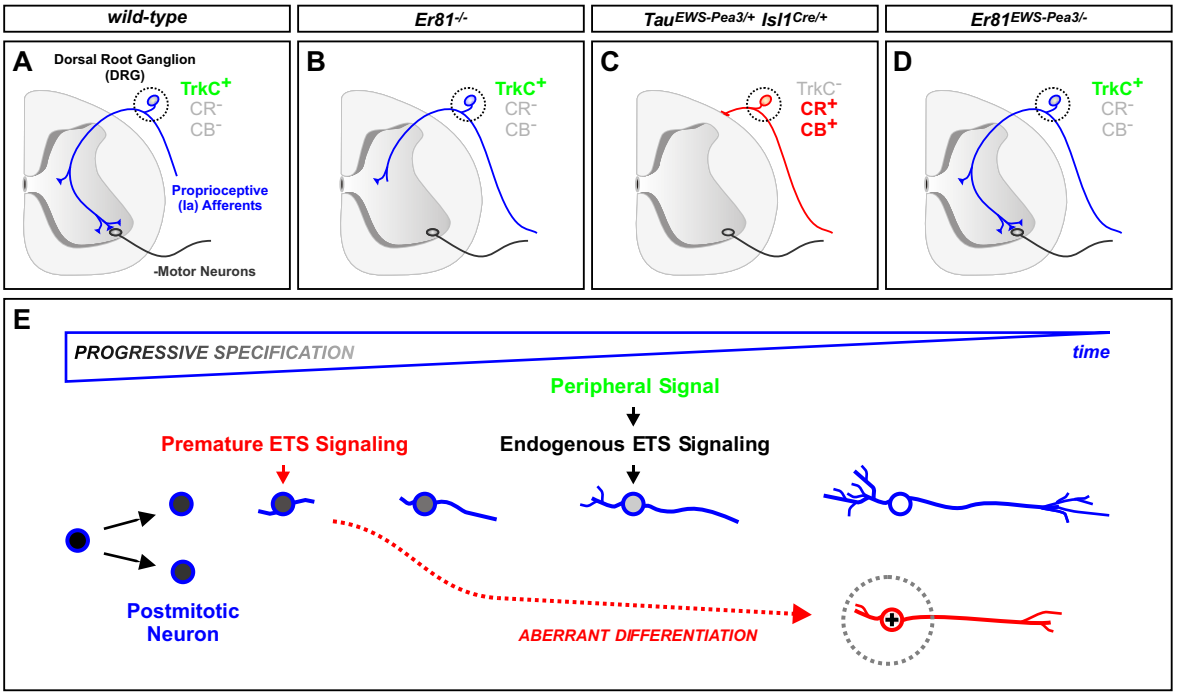


Figure 47. Progressive Neuronal Specification Is Paralleled by a Developmental Shift in Response to ETS Transcription Factor Signaling. Schematic summary diagram illustrating the importance of temporally appropriate upregulation of transcription factor expression during specification of DRG neurons for late aspects of neuronal differentiation and circuit assembly. (A-D) Expression of *EWS-Pea3* from the endogenous *Er81* locus can rescue anatomical defects observed in *Er81*$^{-/-}$ mice and no change in expression of TrkC (green) or Calretinin (CR; grey) and Calbindin (CB; grey) is observed in proprioceptive afferents (A, B, D). In contrast, expression of EWS-Pea3 in early postmitotic DRG neurons leads to severe defects in the establishment of projections accompanied by inappropriate gene expression changes (C; upregulation of CR and CB (red) and downregulation of TrkC (grey)). (E) Premature ETS signaling (red) during progressive specification of proprioceptive sensory neurons leads to aberrant neuronal differentiation (red dashed line). In contrast, the normal target-induced (green; peripheral signal) onset of ETS transcription factor signaling (black) induces appropriate terminal neuronal differentiation (blue).

Molecular Specificity in ETS Transcription Factor Action

In this study, we show that the function of *Er81* in DRG neurons to control development of proprioceptive afferent projections into the ventral spinal cord cannot be substituted for by the highly homologous ETS transcription factor *Pea3* whereas *EWS-Pea3* can replace *Er81*. Since Er81 and Pea3 share DNA binding domains of a high degree of homology (~95% amino acid identity) that have been shown to have very similar *in vitro* DNA binding preferences (Brown and McKnight, 1992; Brown et al., 1998), our findings suggest that the ETS DNA binding domain of both Er81 and Pea3 can function to control development of proprioceptive afferent projections. Moreover, other studies have shown that the break-point fusion protein between EWS and the ETS gene Fli-1 binds DNA with the same sequence specificity as the wild-type Fli-1 protein (Bailly et al., 1994; Mao et al., 1994), suggesting that exchange of the amino-terminal transactivation domain of Pea3 by EWS most likely does not influence the DNA binding preferences of the fusion product EWS-Pea3. We therefore favor the idea that the DNA binding domains of Er81 and Pea3 are interchangeable with respect to their function in promoting proprioceptive afferent growth into the ventral horn of the spinal cord.

There are several possible explanations why the amino-terminal transactivation domain of *Pea3* may not be sufficient to substitute for *Er81* function. First, the amino-terminal region of *Pea3* may not be sufficient to interact with essential transcriptional cofactors expressed specifically in proprioceptive afferents and required to promote axon growth into the ventral spinal cord. In support of this idea, several ETS transcription factors have been described to be activated through relief of autoinhibition upon interaction with cofactors and/or post-translational modifications (Greenall et al., 2001; Pufall and Graves, 2002; Verger and Duterque-Coquillaud, 2002). The amino-terminal fusion with EWS could circumvent a need for interaction with a cofactor and while Pea3 might not be activated by the same cofactors as Er81, EWS-Pea3 would become functionally uncoupled from such an interaction by its amino-terminal domain. Second, the amino-terminal region of Pea3 may harbor insufficient intrinsic transactivation potential within DRG neurons while the fusion with EWS might render Pea3 sufficiently potent to activate the appropriate downstream genes to substitute for Er81 function. In favor of this idea, the results obtained from our cell culture assays in COS-7 cells show that a minimal reporter construct can be activated more efficiently by EWS-Pea3 than by Pea3. These findings do however not exclude the possibility that in a different cellular context such as in DRG neurons, the transcriptional transactivation potential of ETS transcription factors may be different from our minimal cell culture assay. Nevertheless, our findings show that EWS-Pea3 harbors the intrinsic ability to functionally substitute for Er81 within

proprioceptive afferents to control the establishment of projections into the ventral horn of the spinal cord.

Temporal Control of Transcription Factor Activation During Neuronal Differentiation

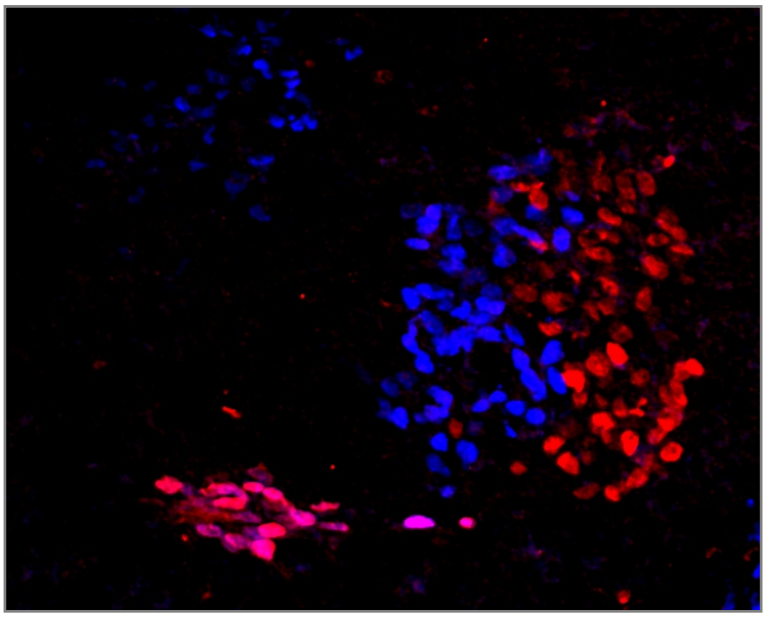
Our findings are compatible with a model in which DRG neurons acquire their mature fate by sequential and temporally-controlled addition of lineage-specific features (Figure 47). At late postmitotic stages, target-derived factors act on pre-determined neuronal lineages to switch their developmental programs to become compatible with processes such as target invasion and branching. Such a transition state in the acquisition of a defined neuronal fate would be accompanied by the induction of appropriate transcriptional programs through the expression of specific transcription factors. Mechanisms such as chromosomal remodeling that restrict or expand access to certain target genes (Kouzarides, 2002) or activation by cofactors responsible to change the action of particular transcription factors (Verger and Duterque-Coquillaud, 2002) could represent possible mechanisms by which the downstream transcriptional profile of a transcription factor could be temporally shifted towards the selection and control of distinct target genes. Our experiments demonstrate a profound change in the action of *EWS-Pea3* at the level of transcriptional regulation within DRG neurons over time. Moreover, this transcriptional shift is paired with the onset of appropriate regulation of neuronal subtype specification and establishment of axonal projections into the target area.

Recent experiments addressing the temporal requirement of transcription factor action in proliferating neural progenitor cells adds to the idea that defined temporal windows during which transcription factors act to control distinct downstream target genes and thus biological functions are of key importance to neuronal fate acquisition. During *Drosophila* neuroblast generation, the transcription factor hunchback controls specification and differentiation of early born neuroblasts (Isshiki et al., 2001). Over time however, neuroblasts progressively lose their competence to generate cells of an early fate in response to hunchback expression (Pearson and Doe, 2003). These findings thus also argue for a change in cellular competence to respond to a specific transcription factor over time, albeit in an early precursor context and by a loss of response rather than a change. More generally, during the differentiation of haematopoietic lineages, several transcription factors have also been shown to exhibit distinct functions at progressive steps of lineage specification (Orkin, 2000). Analysis of the mechanisms by which transcription factor programs can be shifted over time to control different complements of downstream genes and thus aspects of neuronal and

cellular fates in progenitor cells or postmitotic neurons may provide further insight into the way transcription factors act to control the assembly of neuronal circuits.

Chapter 7

Aberrant Differentiation of Spinal Motor Neurons Upon Premature Expression of ETS Activity



INTRODUCTION

The exclusive capability of distinct classes of neurons to assemble into defined neuronal circuits reflects the function of molecular properties that these neurons acquire from the earliest stages on and during their entire differentiation process. Molecular features that distinguish different classes of neurons coordinate cell body migration, direct axonal projections towards the target region, control the precise formation of synaptic connections and shape neurotransmitter identity. The molecular specification of individual subsets of neurons occurs sequentially, involving the progressive restriction in the developmental potential of progenitors as well as postmitotic neurons (Edlund and Jessell, 1999).

Motor neurons in the ventral spinal cord represent one specific class of neurons that are stereotypically interconnected within locomotor circuits controlling body movement (Brown, 1981; Landmesser, 2001; Jacob et al., 2001). Motor neurons arise from initially uncommitted, dividing ventral progenitors in the ventricular layer of the developing neural tube (Jessell, 2000). The topographic and functional organization of spinal motor neurons is established during successive phases of specification and differentiation and correlates with selective patterns of expression of various families of transcription factors (Lee and Pfaff, 2001).

Somatic motor neurons coalesce to form “longitudinal” columns along the entire rostro-caudal body axis, each characterized by a discrete mediolateral position within the spinal cord and the expression of a specific set of LIM-homeodomain (LIM-HD) transcription factors (Tsuchida et al., 1994; Jessell, 2000; Shirasaki and Pfaff, 2002). Furthermore, subpopulations of motor neurons are clustered together into discrete pools innervating distinct limb muscles whereby the pool identity of individual motor neurons can be defined on the molecular level in part by the status of expression of ETS domain transcription factors, notably Er81 and Pea3 (Lin et al., 1998, Sharrocks et al., 2001). The initiation of expression of these two ETS proteins within different motor neuron pools appears to be tightly regulated by the availability of peripheral signals (Lin et al., 1998). Recent studies have shown that induction of expression of Pea3 in the cell bodies of specific motor neuron pools innervating the *latissimus dorsi* and *cutaneous maximus* muscles is regulated through the target derived signal glial cell line-derived neurotrophic factor (GDNF; Haase et al., 2002). Furthermore, expression of Pea3 in these motor neurons is required to control specific late aspects of differentiation conceptually similar to the function of Er81 in proprioceptive DRG sensory neurons (Livet et al., 2002; Arber et al., 2000). These findings together with the results presented in chapter six provide evidence and suggest that the temporally controlled induction of ETS

transcription factors in defined neuronal subpopulations might be of functional importance for the appropriate initiation and execution of genetically late controlled differentiation programs.

This chapter focuses on premature ETS transcriptional signaling in motor neurons and the consequences with respect to motor neuron differentiation. Reasonably, premature early transcription factor expression might also interfere with the endogenous temporally tightly regulated genetic program in motor neurons similar as in DRG neurons. However, it is not clear whether any potential changes in gene expression might occur in an equal or different molecular fashion as previously shown for DRG sensory neurons (for details see chapter six). Motor neurons exposed to premature ETS activity (EWS-Pea3) indeed induced an aberrant differentiation program including downregulation of prominent motor neuron specific markers and neurotrophin receptors accompanied by a reduction in naturally occurring cell death. In addition, motor neurons induced a motor neuron incongruent transcriptional program resulting in the upregulation of inappropriate downstream genes. As a perhaps causal consequence, the columnar organization of somatic motor neurons was severely disturbed. These results taken together with the findings presented in chapter six provide complementary evidence that the sequential initiation of transcription factor expression in postmitotic neurons is required to ensure the adequate adjustment of the transcriptional specification program through the temporally coordinated induction of appropriate target genes at the respective phases during differentiation.

RESULTS

Aberrant Columnar Motor Neuron Organization in Tau$^{EWS\text{-}Pea3}$ Isl1^{Cre} Mutant Mice

A prominent and characteristic feature in the course of motor neuron differentiation is the segregation of somatic spinal motor neurons into two main columns, the medial motor column (MMC) and the lateral motor column (LMC) at limb levels (Jessell, 2000). To begin to address the influence of premature *EWS-Pea3* expression on early postmitotic motor neuron differentiation with respect to the overall topographical organization of the MMC and LMC, whole mount preparations of the spinal cord in *Tau$^{EWS\text{-}Pea3/+}$ Isl1$^{Cre/+}$* mutant were compared to *Tau$^{mGFP/+}$ Isl1$^{Cre/+}$* control embryos.

The conditional activation of *Ews-Pea3* and/or *mGFP* from the genomic *Tau* locus using the *Isl1$^{Cre/+}$* driver (Srinivas et al., 2001) not only promotes efficient expression of *EWS-Pea3* and/or

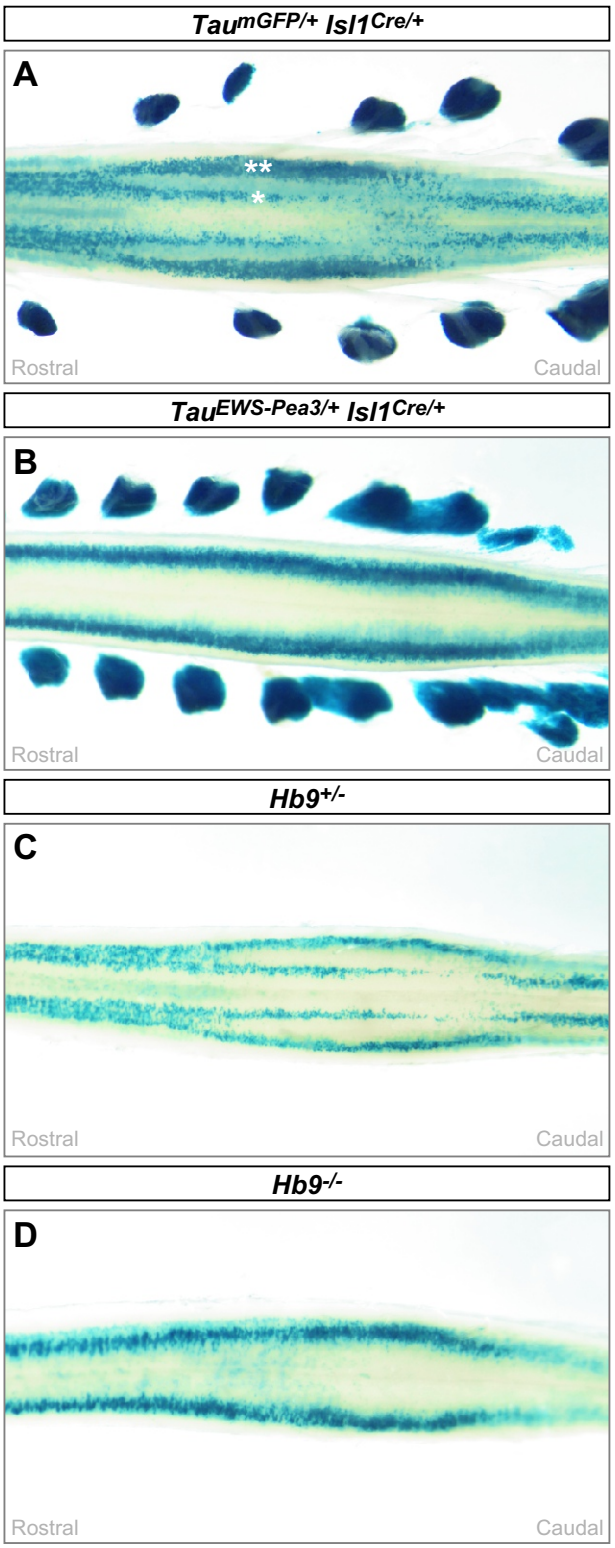


Figure 48. Disorganization of Motor Neuron Columnar Topology in *Tau*$^{EWS\text{-}Pea3}$ Embryos. Ventral view of whole-mount LacZ (Gal activity) staining of E16.5 lumbar spinal cord in *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (A), *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B), *Hb9*$^{+/-}$ (C) and *Hb9*$^{-/-}$ (D). A clear segregation of Gal-labeled cells in MMC (*) and LMC (**) is observed in *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (A) and *Hb9*$^{+/-}$ (C) but not in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B) and *Hb9*$^{-/-}$ (D) embryos.

mGFP in DRG sensory neurons, as described in chapter six, but also in a population of interneurons in the intermediate and in somatic motor neurons in the ventral spinal cord thus allowing the analysis of motor neurons that were exposed to EWS-Pea3 at early postmitotic stages. Furthermore, the insertion of an internal ribosomal entry site followed by *NLS-LacZ* 3' to *EWS-Pea3* or *mGFP* in the *Tau* targeting vectors (see chapter six, Figure 29) allowed tracing of all cells which had undergone recombination. Whole mount preparations of the entire spinal cord were incubated in X-Gal to visualize LacZ expression. Premature expression of EWS-Pea3 in motor neurons led to severe disorganization of all somatic motor columns in the spinal cord. Whereas the clear spatial separation of the MMC and LMC was evident in the control ($Tau^{mGFP/+}$ $Isl1^{Cre/+}$; Figure 48A), mutants expressing *EWS-Pea3* ($Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$; Figure 48B) displayed no obvious separation of MMC and LMC at lumbar levels. Rather, the motor neurons seemed to form a single continuous motor column extending from the most rostral cervical to caudal segments without any signs of obvious segregation into a medial and lateral motor column at brachial and lumbar limb levels (Figure 48, data not shown). Interestingly, a very similar phenotype is observed when the homeodomain transcription factor Hb9 is ablated (Figure 48C, D; see also Arber et al., 1999; Thaler et a., 1999). Thus, temporally ectopic premature expression of EWS-Pea3 in early postmitotic motor neurons results in a topographically aberrant columnar organization phenotype similar to the one observed in $Hb9^{-/-}$ mutant embryos (Figure 48; Arber et al., 1999; Thaler et al., 1999).

Early Expression of EWS-Pea3 in Motor Neurons Results in Downregulation of Hb9

Expression of Hb9 represents a key hallmark in the differentiation process of all motor neurons in the spinal cord and has been shown to be required for the consolidation of the motor neuron identity as well as for the proper segregation of motor neurons into the MMC and LMC (Arber et al., 1999; Thaler et al., 1999). The similarity of the columnar phenotype in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ and $Hb9^{-/-}$ mutants could point towards a genetic interaction between *EWS-Pea3* and *Hb9*. Alternatively, different molecular pathways might be affected when either EWS-Pea3 is present or Hb9 absent in early postmitotic motor neurons. To begin to distinguish between these two possibilities, expression of Hb9 in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant embryos was analyzed using immunohistochemistry. Upon induction of *EWS-Pea3* in early motor neurons using the $Isl1^{Cre/+}$ driver Hb9 expression in motor neurons was downregulated significantly when compared to wild-type (Figure 49A, B). Hb9 expression was not completely absent but expressed only at very low levels in the vast majority of $LacZ^{+}$ cells (Figure 49C, D). The expression of Isl1 is not uniformly

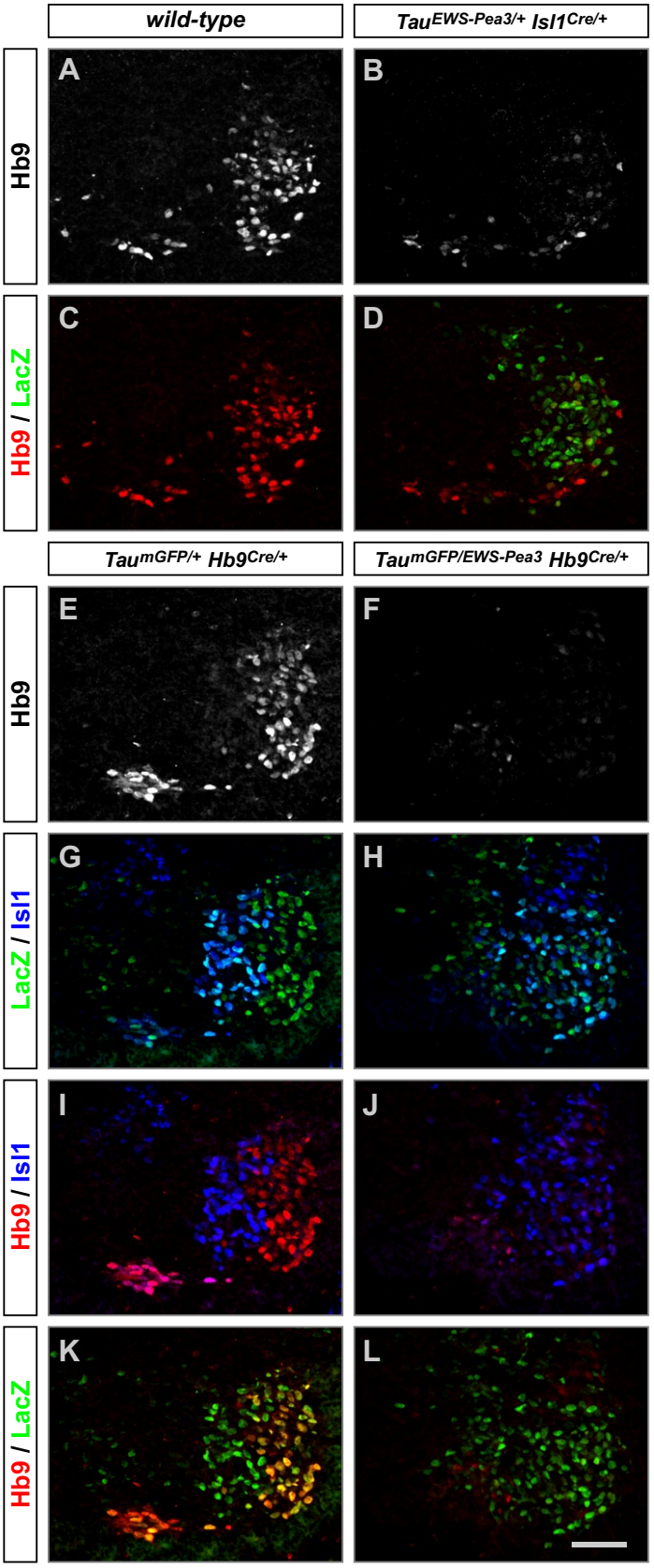


Figure 49. Expression of Hb9 is Downregulated in the Presence of EWS-Pea3 in Motor Neurons. Expression of Hb9 (white: A, B, E, F; red: C, D, I-L), LacZ (green: C, D, G, H, K, L) and Isl1 (blue: G-J) in brachial motor neurons in wild-type (A, C), *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D), *Tau*$^{mGFP/+}$ *Hb9*$^{Cre/+}$ (E, G, I, K) and *Tau*$^{mGFP/EWS\text{-}Pea3}$ *Hb9*$^{Cre/+}$ (F, H, J, L) embryos at E13.5. Scale bar = 60 m.

maintained in the whole motor neuron population (Figure 49G, I; Tsuchida et al., 1994; Arber et al., 1999; Kania et al., 2000) and therefore conditional gene expression using the *Isl1*$^{Cre/+}$ driver might not reach 100% within the entire complement of motor neurons at limb levels (see Experimental Procedures for discussion of this issue). To achieve recombination in a higher number of motor neurons, the *Hb9*$^{Cre/+}$ driver (Yang et al., 2001) was crossed to the *Tau*$^{EWS\text{-}Pea3/+}$ and *Tau*$^{mGFP/+}$ alleles, respectively. Analysis of the expression of Hb9 in *Tau*$^{mGFP/EWS\text{-}Pea3}$ *Hb9*$^{Cre/+}$ revealed that Hb9 was almost completely downregulated in all LacZ positive motor neurons when compared to *Tau*$^{mGFP/+}$ *Hb9*$^{Cre/+}$ (Figure 49E-L) similar the situation observed in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ mutant mice (Figure 49A-D). In summary, these findings could suggest that EWS-Pea3 downregulates the expression of Hb9 either directly or indirectly in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ and *Tau*$^{EWS\text{-}Pea3/+}$ *Hb9*$^{Cre/+}$ mutant embryos, respectively.

Neurotrophin-Independent Survival of Motor Neurons ?

DRG sensory neurons become independent from neurotrophin signaling for survival upon premature expression of EWS-Pea3. This phenotype seems to rely specifically on the early timing of expression of EWS-Pea3 and in addition there is evidence that this phenotype appears in a cell autonomous regulated manner within DRG sensory neurons (see chapter six for details). These findings raise the question whether a similar effect could be attributed to EWS-Pea3 when expressed in early postmitotic motor neurons. To address this issue, expression of neurotrophin receptor components involved in the GDNF signaling cascade (Airaksinen and Saarma, 2002) was analyzed in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ embryos by in situ hybridization because some motor neurons depend on GDNF signaling for survival during the period of naturally occurring cell death (Henderson et al., 1998). The GDNF family of receptors is composed of complexes involving the transmembrane Ret tyrosine kinase and one of four glycosyl phosphatidyl-inositol (GPI) membrane-anchored ligand-binding components, Gfrα1-4. The four GDNF family ligands all use Ret but have their own preferred co-receptors (eg. GDNF binds Gfrα1; Baloh et al., 2000). Expression of both *Gfrα1* and *Ret* was drastically reduced in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ at E13.5 (Figure 50A, B, E, F) and E16.5 (Figure 50C, D, G, H) in comparison to wild-type embryos. These findings raise the question whether this downregulation of expression of neurotrophic receptors has an influence on naturally occurring cell death. Using TUNEL on motor neuron sections it was found that apoptosis was significantly decreased in motor neurons of *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ mutants in comparison to wild-type at E13.5 (Figure 50I, J). Thus, these observations indicate that not only

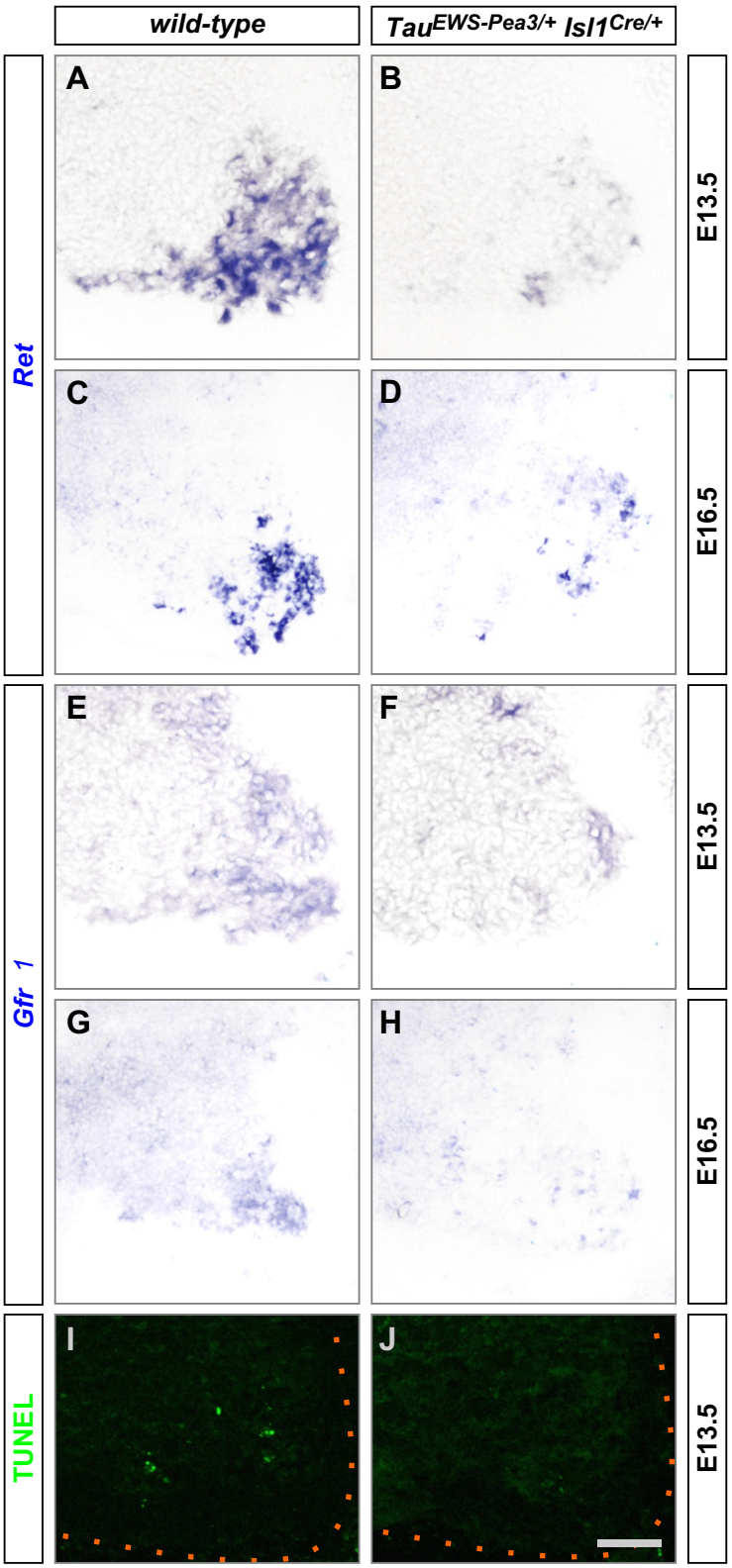


Figure 50. Loss of Expression of GDNF-Signaling Receptor Components and Increased Survival in Motor Neurons of *Tau*$^{EWS\text{-}Pea3}$ Embryos. (A-H) *In situ* hybridization analysis of *Ret* (A-D) and *Gfr 1* (E-H) expression in brachial motor neurons in wild-type (A, C, E, G) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D, F, H) embryos at E13.5 (A, B, E, F) and E16.5 (C, D, G, H). (I, J) Analysis of neuronal cell death in E13.5 brachial motor neurons in wild-type (I) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (J) embryos using TUNEL (green). The ventro-lateral border of the spinal cord is outlined by orange dots. Note the absence of apoptotic figures in *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ mutant embryos (J). Scale bar: (A, B, E, F, I, J) = 50 m; (C, D, G, H) = 80 m.

DRG sensory neurons but also motor neurons tend to survive independent of neurotrophic growth factor signaling upon premature expression of EWS-Pea3.

Cell Fate Acquisition of EWS-Pea3 Expressing Motor Neurons is Affected

Early expression of EWS-Pea3 in DRG sensory neurons interferes with the acquisition of classical DRG sensory neuron fates. The results described in chapter six provide an indication that an alternative inappropriate genetic program is initiated affecting the differentiation of sensory neurons in two ways. First, the vast majority of known cutaneous and proprioceptive specific markers are downregulated. Second, various genes are ectopically upregulated. Both events together result in a divergent differentiation path which DRG sensory neurons follow towards an exclusive and aberrant cell fate that is never observed among wild-type DRG sensory neurons. However, it is not entirely clear how direct EWS-Pea3 could control this shift in the genetic program. Nevertheless, these findings might suggest that premature EWS-Pea3 expression also might interfere with appropriate cell fate acquisition of motor neurons. Therefore, expression of three prominent motor neuron specific markers *Islet1* (*Isl1*), *Choline Acetyltransferase* (*ChAT*), and *Retinaldehyde dehydrogenase-2* (*RALDH-2*) was analyzed by in situ hybridization. The level of expression of all these three markers was reduced to a significant extent on the mRNA level in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant embryos when compared to wild-type at E13.5 (Figure 51) suggesting that indeed motor neurons fail to acquire their appropriate cell fate in the presence of EWS-Pea3.
In addition, the status of expression of some genes and/or their protein products that were ectopically induced in DRG upon premature EWS-Pea3 was followed as well in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant motor neurons. In situ hybridization analysis at E16.5 revealed ectopic expression of the neuropeptide Substance P (*Tachykinin*, Figures 52A, B), the homeodomain transcription factor *Pbx3b* (Figure 52C, D), and the secreted frizzled related protein *sFRP2* (Figure 52E, F) in motor neurons in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant embryos when compared to the wild-type. Furthermore, expression of the two calcium binding proteins calretinin and calbindin was also ectopically induced in motor neurons as revealed by immunohistochemistry at E13.5 (Figures 53, 54). Nevertheless, to prove that ectopic expression of Calretinin, Calbindin and Substance P effectively occurred in EWS-Pea3 positive cells, recombined cells were traced by the expression of LacZ in mutant ($Tau^{mGFP/EWS\text{-}Pea3}$ $Isl1^{Cre/+}$ or $Tau^{mGFP/EWS\text{-}Pea3}$ $Hb9^{Cre/+}$) and control ($Tau^{mGFP/+}$ $Isl1^{Cre/+}$ or $Tau^{mGFP/+}$ $Hb9^{Cre/+}$) embryos at E13.5 (Figures 53, 54). Detailed analysis revealed that not all cells that were positive for LacZ co-expressed either Calbindin, Calretinin or Substance P. In fact, Calbindin, Calretinin and Substance P were induced only in a fraction of motor neurons in

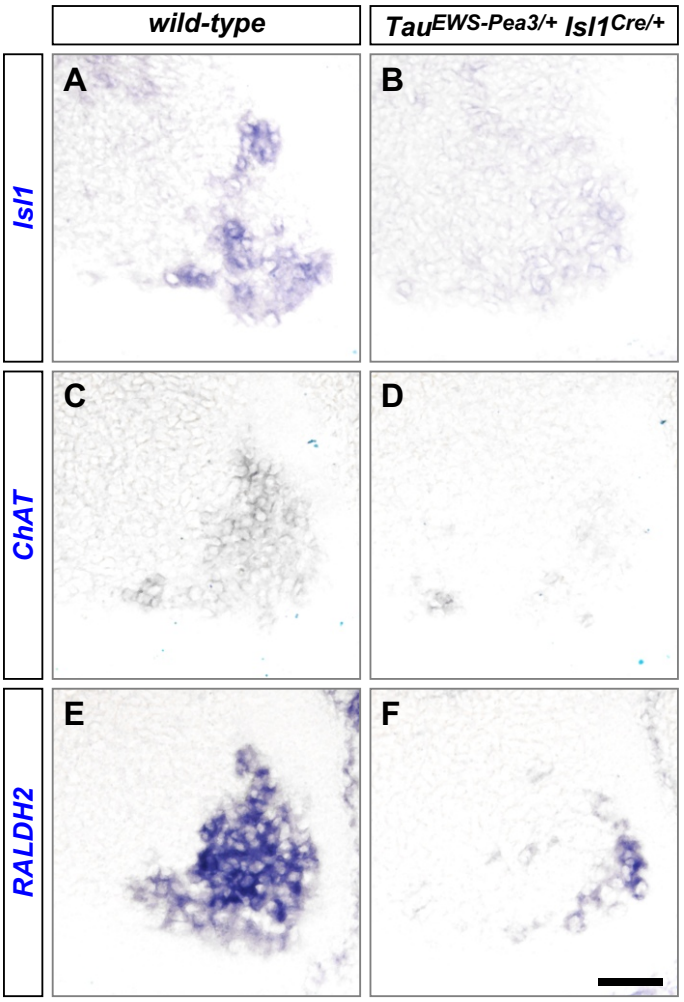


Figure 51. Gene Expression Analysis upon Induction of EWS-Pea3 in Early Postmitotic Motor Neurons - Downregulation. *In situ* hybridization analysis of *Isl1* (A, B), *ChAT* (C, D) and *RALDH2* (E, F) expression in brachial motor neurons of wild-type (A, C, E) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D, F) embryos at E13.5. Scale bar = 35 m.

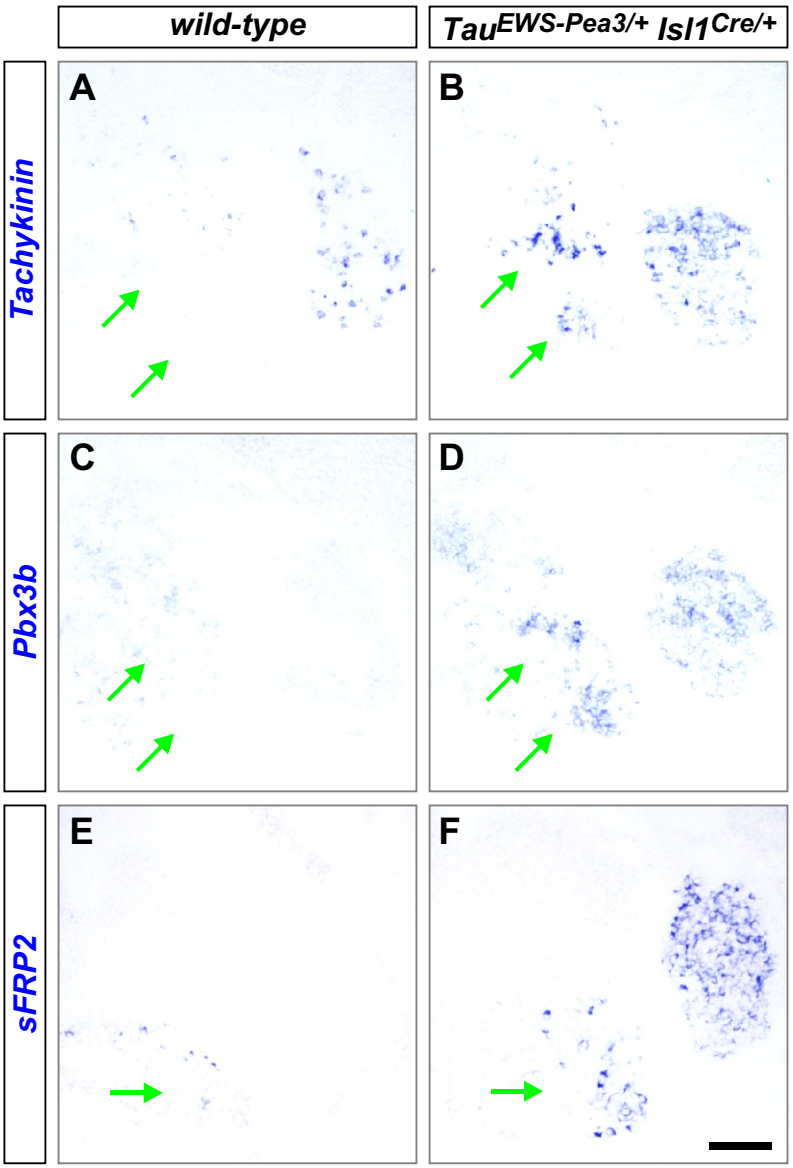


Figure 52. Gene Expression Analysis upon Induction of EWS-Pea3 in Early Postmitotic Motor Neurons - Upregulation. *In situ* hybridization analysis of *Tachykinin* (A, B), *Pbx3b* (C, D) and *sFRP2* (E, F) expression in lumbar motor neurons of wild-type (A, C, E) and *Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D, F) embryos at E16.5. Green arrows point to motor neurons that ectopically upregulate *Tachykinin*, *Pbx3b*, and *sFRP2*, respectively in mutant (*Tau*$^{EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$) but not wild-type embryos. Scale bar = 60 m.

mutant embryos. There was however no co-expression of any of these three proteins and LacZ observed in motor neurons in control embryos indicating that at least a subset of motor neurons might differentiate and acquire a quite similar neuronal cell fate as DRG sensory neurons expressing the EWS-Pea3 fusion protein from early postmitotic stages on.

Peripheral Innervation of Limb Muscles in $Tau^{EWS\text{-}Pea3}$ $Isl1^{Cre}$ Mutant Mice

Sensory neurons that differentiate towards an aberrant cell fate upon premature expression of EWS-PEA3 have been shown to display severe axonal projection defects into the spinal cord as well as to peripheral targets (see chapter six, Figures 30, 31). To begin to assess whether motor neurons that express early EWS-Pea3 display similar defects in the establishment of peripheral projections into limb muscles, axonal terminals in the region of the synaptic endplate band were visualized using immunohistochemistry. Analysis of innervation of limb muscles at late embryonic stages was performed on transverse sections using the Tau^{mGFP} allele and GAP-43 expression to visualize peripheral motor neuron axons, and α-bungarotoxin (BTX) labeling to delinate clusters of acetylcholine receptors (AChR) within the synaptic endplate band in mutant ($Tau^{mGFP/EWS\text{-}Pea3}$ $Isl1^{Cre/+}$) and control ($Tau^{mGFP/+}$ $Isl1^{Cre/+}$) embryos at E16.5 (Figure 55A-D). Furthermore, terminal Schwann cells expressing S100 were visualized (Figure 55E, F). Despite profound deficits were observed in motor neuron differentiation, no obvious defects in the pattern of motor neuron innervation or AChR clustering could be detected in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ at E16.5 (Figure 55). These findings provide evidence that the aberrant cell fate acquired by EWS-Pea3^{+} motor neurons does not interfere with motor axons projecting to the synaptic endplate band within skeletal limb muscles, does however not exclude the possibility that fine aspects of motor neuron innervation at the NMJ might display deficits.

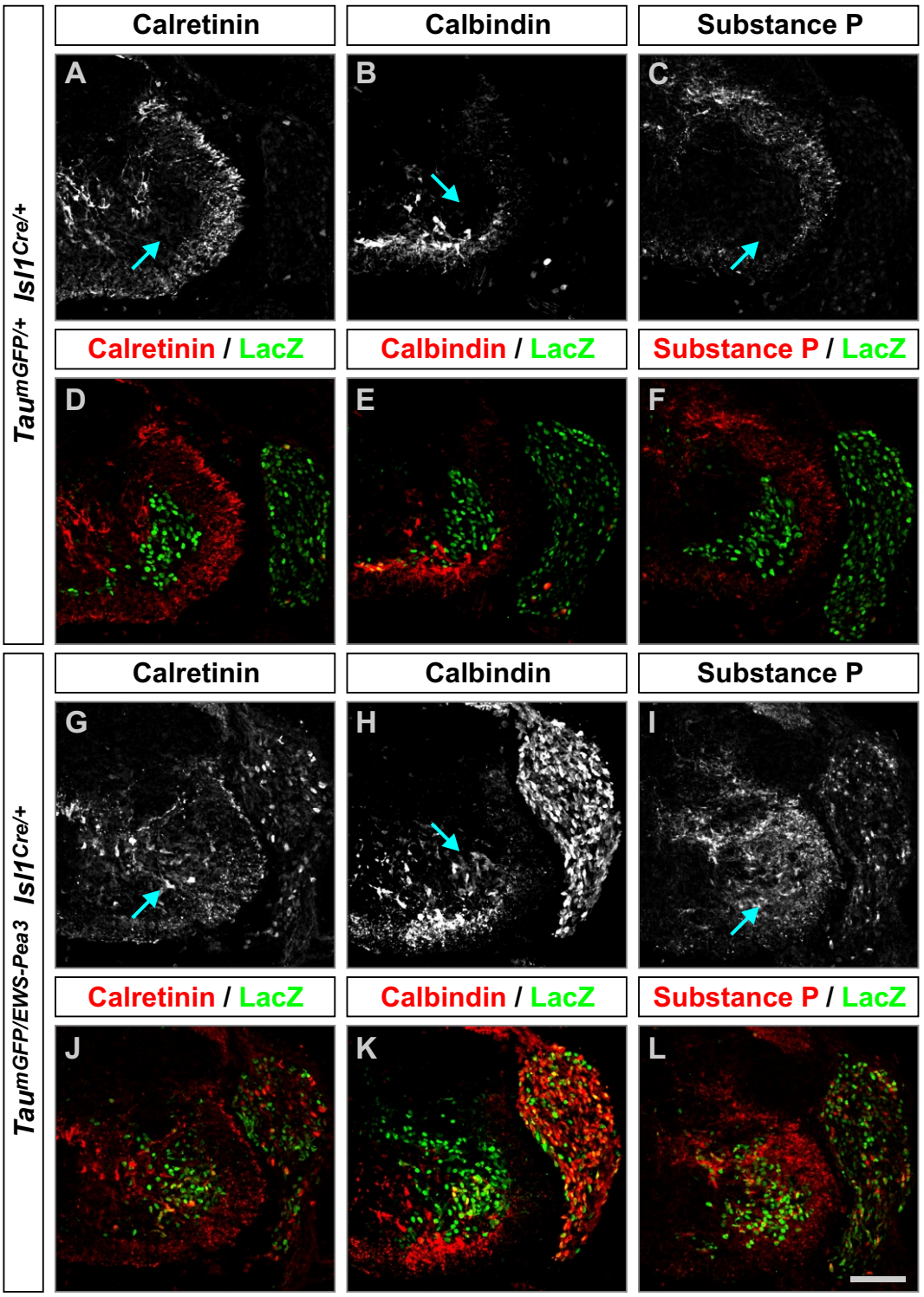


Figure 53. Aberrant Upregulation of Calretinin, Calbindin and Substance P in Motor Neurons of *Tau*$^{EWS\text{-}Pea3}$ *Isl1*^{Cre} Embryos. Analysis of Calretinin (white: A, G; red: D, J), Calbindin (white: B, H; red: E, K), Substance P (white: C, I; red: F, L) and LacZ (green: D-F, J-L) expression in the ventral horn of E13.5 spinal cord of *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (A-F) and *Tau*$^{mGFP/EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (G-L) embryos. Blue arrows point to motor neurons that ectopically upregulate Calretinin, Calbindin and Substance P, respectively, in mutant (*Tau*$^{mGFP/EWS\text{-}Pea3}$ *Isl1*$^{Cre/+}$) but not control (*Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$) embryos. Scale bar = 85 m.

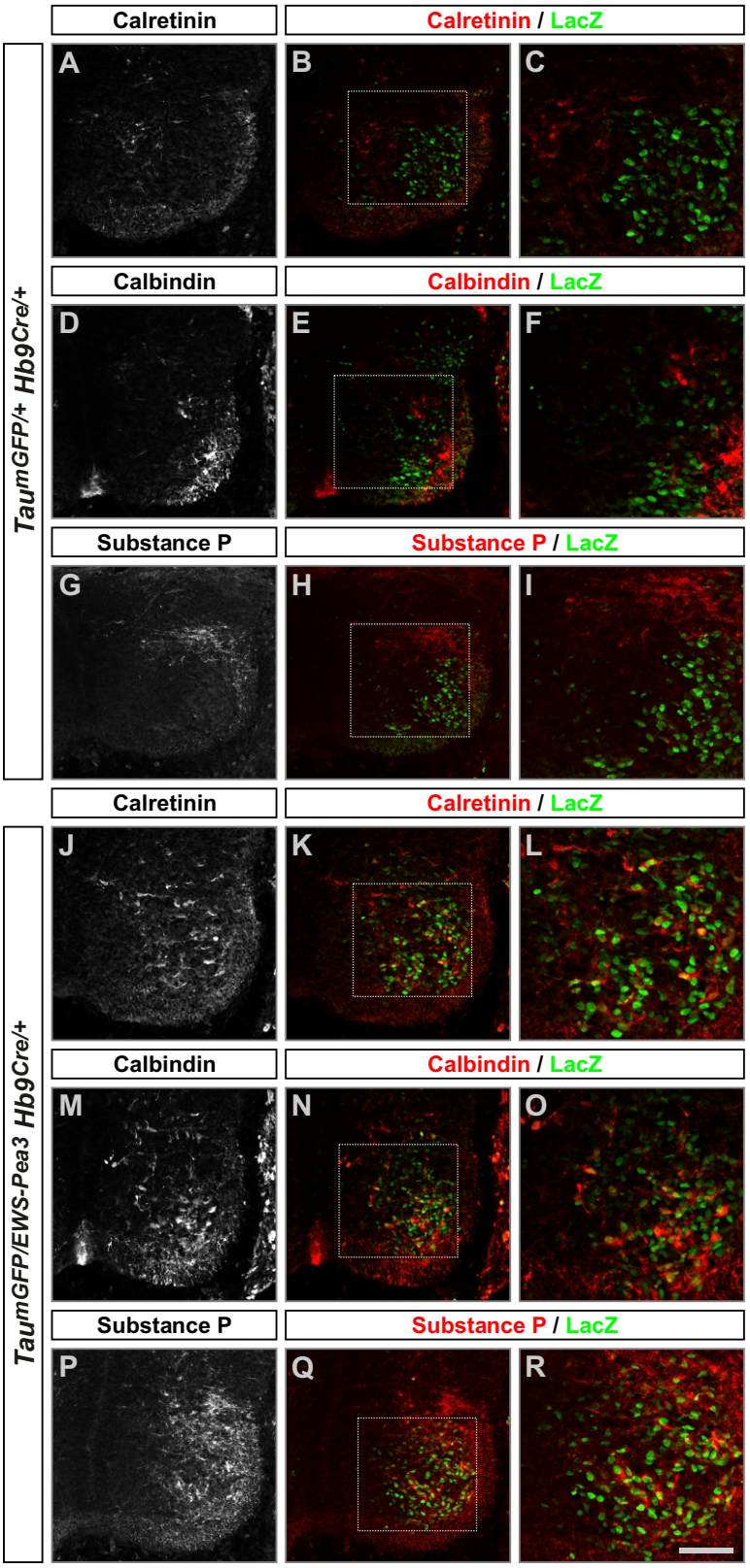


Figure 54. Aberrant Upregulation of Calretinin, Calbindin and Substance P in Motor Neurons of *Tau$^{EWS\text{-}Pea3}$ Hb9^{Cre}* Embryos. Analysis of Calretinin (white: A, J; red: B, C, K, L), Calbindin (white: D, M; red: E, F, N, O), Substance P (white: G, P; red: H, I, Q, R) and LacZ (green: B, C, E, F, H, I, K, L, N, O, Q, R) expression in the ventral horn of E13.5 spinal cord of *Tau$^{mGFP/+}$ Hb9$^{Cre/+}$* (A-I) and *Tau$^{mGFP/EWS\text{-}Pea3/+}$ Hb9$^{Cre/+}$* (J-R) embryos. Boxes in (B, E, H, K, N, Q) indicate views shown at higher magnification in (C, F, I, L, O, R) Scale bar: (A-H) = 80 m; (I-L) = 40 m.

DISCUSSION

Together with the findings presented in the previous chapter six, the results described within this chapter contribute to the emergence of a concept for a temporal switch in the competence of transcription factor signaling to induce defined cellular responses during defined successive phases within the specification and differentiation process of discrete subsets of postmitotic neurons. The experimental data provide evidence that the EWS-Pea3 fusion protein seems to harbor the intrinsic capacity to shift at least two independent populations of early postmitotic neurons within distinct cellular contexts towards an aberrant cell fate characterized by neurotrophin independent survival, downregulation of cell specific markers, and induction of an inappropriate differentiation program. The alteration of the motor neuron cell fate as a consequence of premature EWS-Pea3 as well as a comparison of the premature EWS-Pea3 evoked phenotype in motor versus DRG sensory neurons will be the subjects of the following discussion.

Transcription Factors and Topographical Motor Neuron Differentiation

Particular subsets of motor neurons require target derived GDNF to induce endogenous expression of Pea3 that in turn regulates late differentiation steps including terminal cell body settling and target invasion (Livet et al., 2002; Haase et al., 2002). Premature expression of the EWS-Pea3 chimera, representing a transcriptionally more potent variant of Pea3 results in an aberrant cell fate distinct from a 'normal' Pea3 positive motor neuron phenotype. One feature of this inappropriate cell fate might be a cell migration defect since the formation and segregation of motor neurons into a MMC and LMC is blocked in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mice. Interestingly, as consequence of premature expression of EWS-Pea3, expression of the transcription factor Hb9 is downregulated in cells that encountered EWS-Pea3. In turn, topographic organization of motor neuron differentiation in the spinal cord is affected in $Hb9^{-/-}$ mutants (Arber et al., 1999; Thaler et al., 1999) in a strikingly similar way as observed in $Tau^{EWS\text{-}Pea3}$ mutant mice.

Although it is not clear how direct or indirect the action of EWS-Pea3 affects Hb9 expression, the absence of Hb9 in motor neurons upon early expression of EWS-Pea3 could provide at least in part a molecular explanation for the observed disorganization of the motor columns in $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ mutant mice. Nevertheless, the possibility that EWS-Pea3 on its own could actively contribute to promotion of aberrant columnar organization cannot be excluded at this level of analysis for the following reason. Native Pea3 has been shown to fulfill an essential role in motor neuron pool clustering presumably through the regulation of expression of certain Type II

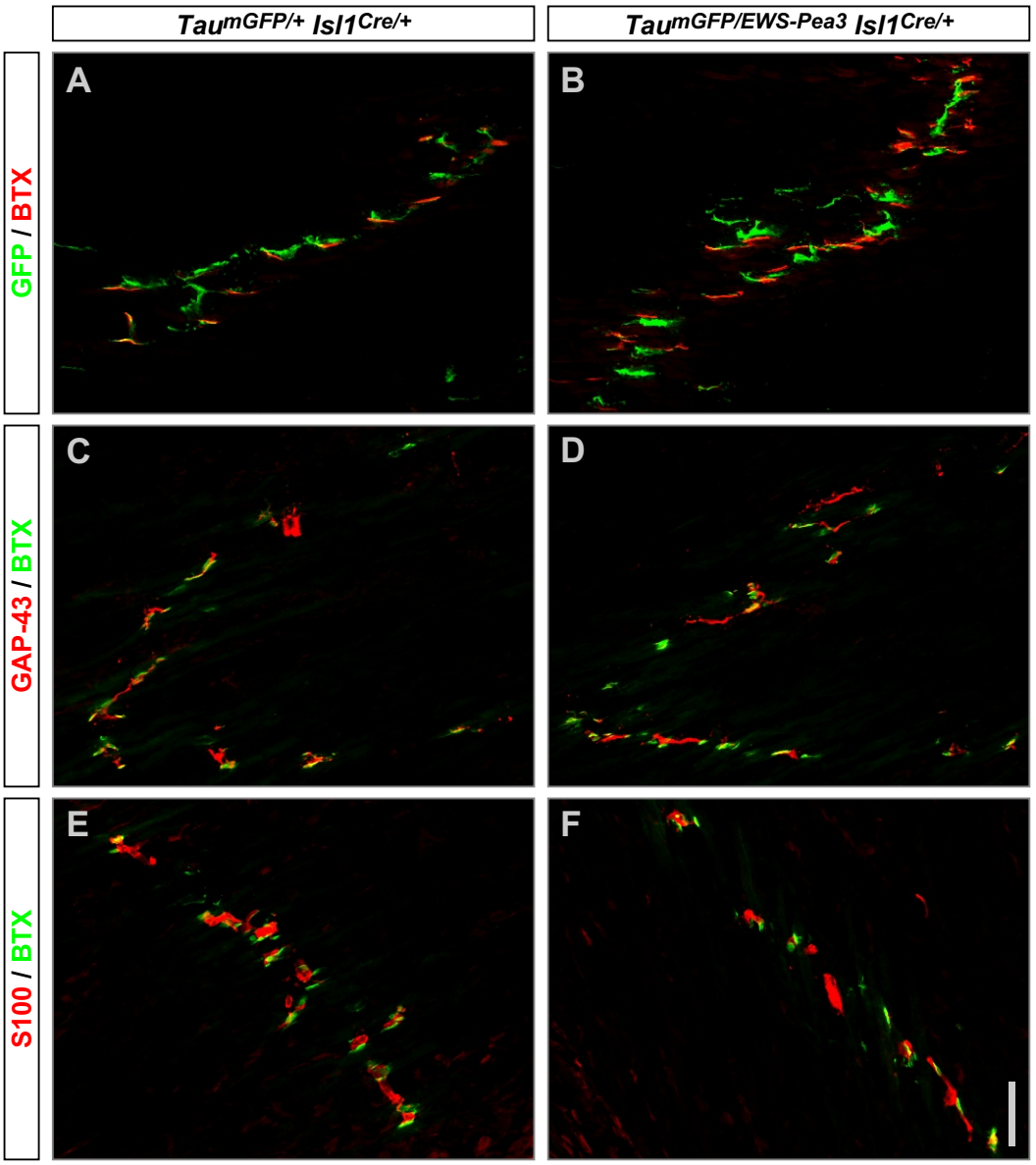


Figure 55. Peripheral Motor Axon Projections in *Tau*$^{EWS\text{-}Pea3}$ Embryos. (A-D) Double-label immunohistochemical detection of motor neuron axonal terminals by Cre recombinase mediated activation of mGFP expression (A, B: green) from the *Tau* locus or expression of GAP-43 (C, D) and -bungarotoxin-labeled AChR clusters (BTX; A, B: red; C, D: green) in hindlimb muscles of *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (A, C) and *Tau*$^{mGFP/EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (B, D) embryos at E16.5. (E, F) Double-label immunohistochemical detection of S100^{+} Schwann cells (red) and -bungarotoxin-labeled AChR clusters (BTX; green) in hindlimb muscles of *Tau*$^{mGFP/+}$ *Isl1*$^{Cre/+}$ (E) and *Tau*$^{mGFP/EWS\text{-}Pea3/+}$ *Isl1*$^{Cre/+}$ (F) embryos at E16.5. Scale bar = 50 m.

cadherins and semaphorins which in turn might contribute to correct motor pool positioning (Livet et al., 2002; Price et al., 2002). In addition, transcriptional activation assays have demonstrated that EWS-Pea3 represents a more potent, but ETS DNA-binding site specific, transcriptional activator than native Pea3 *in vitro* (details are shown in chapter six, Figure 27A). Therefore, it is tempting to speculate that the broad expression of EWS-Pea3 in early $Tau^{EWS\text{-}Pea3/+}$ $Isl1^{Cre/+}$ motor neurons might initiate a transcriptional program resulting in a temporal and spatial deregulation of genes encoding cadherins and/or semaphorins that normally could play a prominent role in $Pea3^{+}$ motor neuron pool formation. This would however implicate that probably a subset of these genes are also downstream of Hb9 but repressed in the wild-type situation when considering the common columnar phenotype in $Tau^{EWS\text{-}Pea3/+}$ and $Hb9^{-/-}$ mutant mice. It would be interesting to analyze potential motor neuron specific target genes of all three Hb9 and EWS-Pea3 in comparison to native Pea3 using Affymetrix gene chip technology since this approach might potentially lead to the identification of additional genes, besides the known LIM-HD proteins, controlling the early topographical organization of motor neurons into the medial or the lateral motor column. Furthermore, genes, besides ETS and type II cadherins, which play an essential role in the clustering and organization of distinct motor neuron pools, might be identified by such an approach.

EWS-Pea3^{+} Motor Neurons vs. EWS-Pea3^{+} DRG Sensory Neurons

In the course of postmitotic differentiation, motor neurons share various aspects with DRG sensory neurons in terms of molecular signaling principles. In particular, survival of both, motor and DRG sensory neurons is under strict peripheral neurotrophic control (Henderson et al., 1998; Bibel and Barde, 2000; Huang and Reichardt, 2003). In addition, distinct subpopulations of both motor and sensory neurons appear to undergo a temporal switch with respect to the respective differentiation program and change from axonal growth and elongation towards target invasion, branching and arborization as soon as axonal growth cones reach the target region. Recent evidence might suggest that this switch in differentiation could be regulated or mediated through induction of specific timely appropriate transcriptional programs. In particular target-derived neurotrophin NT-3 signals retrogradely to the cell body of proprioceptive Ia afferents to induce expression of Er81 (Patel et al., 2003). Conversely, target-derived GDNF leads to induction of Pea3 in motor neurons (Haase et al., 2002). Both of these ETS genes Er81 and Pea3 have been shown to regulate late aspects of differentiation of proprioceptive and motor neurons (Arber et al., 2000; Livet et al., 2002), respectively, after this ‘magic’ switch in transcriptional competence has occurred. The conclusions

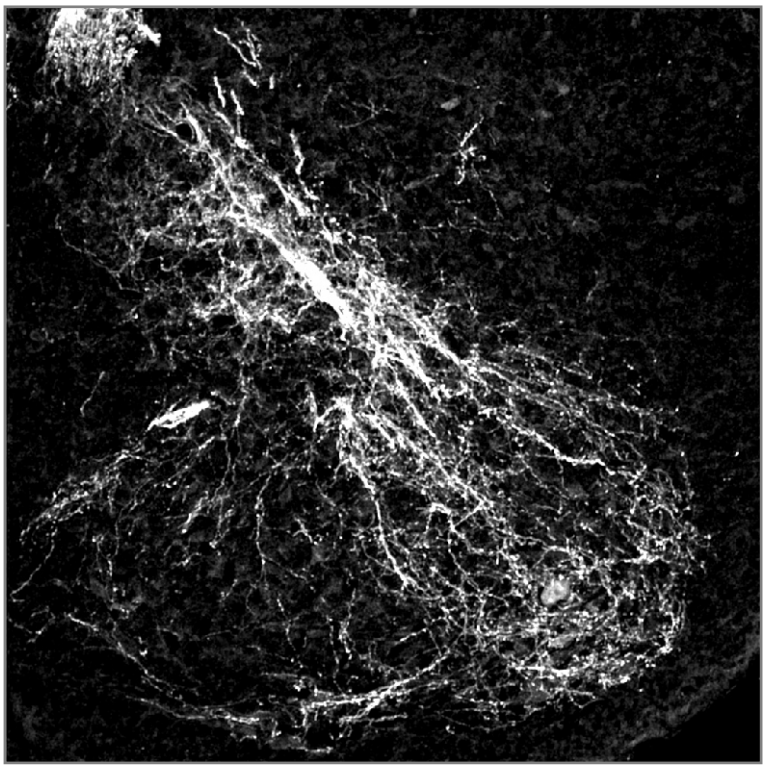
drawn from chapter six allows the notion that a switch is most likely required to render (at least) DRG sensory neurons competent to respond appropriately to ETS signaling. Premature induction of ETS transcriptional activity leads to the induction of an aberrant differentiation program paired with inadequate gene expression profiles, including downregulation of neurotrophin receptors in DRG sensory neurons. In a conceptually similar or related manner, premature expression of ETS transcriptional activity in motor neurons also results in downregulation of the neurotrophin receptor Gfrα1 and the co-receptor Ret highlighting an interesting parallel to DRG sensory neurons. Furthermore, genes that were ectopically induced in DRG sensory neurons upon premature ETS signaling were also found to be upregulated in motor neurons that encountered early ETS activity. Two possibilities might account for these similar changes in gene expression. First, the genes in question are direct ETS target genes and could be upregulated by default in response to ETS expression. Second, motor and sensory neurons could share similar but maybe not identical 'competence' with respect to the regulation of the global gene expression program. However, both mechanisms might act in parallel but probably not equally in both DRG sensory and spinal motor neurons, because not all recombined cells that express premature ETS activity in the spinal cord upregulate calbindin and calretinin whereas nearly every recombined DRG sensory neuron upregulates these two calcium binding proteins. Furthermore, it is not clear whether all changes in gene expression are mediated exclusively by EWS-Pea3. The possibility that immediate EWS-Pea3 downstream transcriptional regulators specific either for motor or DRG sensory neurons might contribute to the acquisition of the observed respective cell fates should also be taken into account.

With respect to axonal growth and pathfinding as a measure of a defined biological response upon premature ETS activity, DRG sensory neurons appeared to display more severe deficits than motor neurons. These observations might provide an indication that the premature induction of EWS-Pea3 differentially affects the growth and/or axonal arborization program in DRG and motor neurons, respectively.

Together, the experimental data described in this and the previous chapter six begins to establish a concept and hypothesis where the temporal control and induction of specific transcriptional programs in postmitotic neurons represents a central aspect. It will be very exciting to resolve the exact time frame within which specific transcription factors change the transcriptional genetic profile of particular neuronal populations at defined temporal stages of differentiation to control appropriate cellular maturation.

Chapter 8

ETS Transcription Factor Specificity in Sensory-Motor Connectivity



INTRODUCTION

Precise control in the course of selective synapse formation is essential for correct interconnection of neurons and their appropriate assembly into neuronal circuits throughout the nervous system. The classical monosynaptic stretch reflex circuit represents a relative simple neuronal circuit as two neuronal elements, a motor and sensory unit are sufficient to close this "hard-wired" circuit through a monosynaptic connection within the spinal cord (Eccles et al., 1957; Brown, 1981, Glover, 2000). The motor unit includes α-motor neurons with their cell bodies located in the ventral horn of the spinal cord that project out of the central nervous system towards specific target muscles to innervate extrafusal muscle fibers in the region of the synaptic endplate band at neuromuscular junctions (NMJ) (Landmesser, 2001). The sensory unit is represented by proprioceptive Ia afferent sensory neurons (Chen et al., 2003). The cell bodies of Ia afferents are located within the dorsal root ganglia (DRG) arrayed along the rostro-caudal axis on either side of the spinal cord at each segmental level. Ia afferents extend two axonal branches whereby the peripheral ones innervate muscle spindles, specialized sensory organs, composed of intrafusal muscle fibers and embedded stereotypically among the extrafusal muscle fibers within particular limb muscles (Zelena, 1994; Maier, 1997). The central branches of Ia afferents project into the spinal cord, arborize extensively towards the ventral termination zone and form direct monosynaptic connections with α-motor neurons to convey information about the state of muscle contraction back to the central nervous system (Brown, 1981; Chen and Frank, 1999; Chen et al., 2003).

The generation, early specification and initial cell fate acquisition of proprioceptive DRG sensory neurons requires a specific genetic transcriptional program shaped by the proneural basic helix loop helix (bHLH) Neurogenin2 (Ngn2) and the Lim-homeodomain protein Islet1 (Isl1) amongst other transcriptional regulators (Ma et al., 1999; Pfaff et al., 1996). However, the knowledge of the identity of molecular cascades at work directly downstream of these factors remains largely elusive. Nevertheless, proprioceptive sensory neurons specifically express the neurotrophin receptor TrkC (Klein et al., 1994). Periphery derived NT-3, signaling via TrkC, is essential during the period of naturally occurring cell death to prevent apoptosis (Ernfors et al., 1994; Klein et al., 1994; Bibel and Barde, 2000; Huang and Reichardt, 2003) but also involved in the initiation of distinct late aspects of proprioceptive sensory neuron differentiation program (Patel et al., 2003). In particular, NT-3 signaling is required for the induction of the ETS transcription factor Er81 (Patel et al., 2003) at a stage at which proprioceptive Ia afferents seem to undergo a switch in their developmental program from axon growth and elongation towards branching, arborization into the target area and

the initiation of synaptogenesis. At these late phases in the course of proprioceptive specification and differentiation Er81 expression and/or activity in Ia afferents is required for the promotion of axonal projections and arborization into the ventral horn of the spinal cord (Arber et al., 2000). In the absence of Er81 expression in proprioceptive DRG neurons in either *Er81*$^{-/-}$ mutant or *NT-3*$^{-/-}$ *Bax*$^{-/-}$ double mutant mice Ia afferents fail to elaborate a ventral termination zone and as a consequence no monosynaptic connections are formed with α-motor neurons (Arber et al., 2000; Patel et al., 2003). These findings demonstrate that Er81, being a member of the small Pea3 subfamily of ETS transcription factors that also includes the highly homologous Pea3 and Erm proteins (Sharrocks, 2001), fulfills an essential role in late steps of specification and differentiation of proprioceptive Ia afferents.

Another transcription factor Runx3, member of the runt family (Levanon and Groner, 2004), was shown to be expressed specifically in proprioceptive sensory neurons in the vast majority of Er81 positive DRG cells (Levanon et al., 2002; Inoue et al., 2002; I. Kramer and S. Arber, personal communication). Interestingly, ablation of Runx3 results in two variable phenotypes, probably depending on the mouse genetic strain background. While Levanon et al. observed a dramatic loss of TrkC positive proprioceptive neurons in DRG of *Runx3*$^{-/-}$ mutants, Inoue and colleagues reported a *Runx3*$^{-/-}$ phenotype sharing numerous characteristic aspects with the *Er81*$^{-/-}$ phenotype but no reduction in the TrkC population of DRG sensory neurons.

In summary, as a consequence of ablation of either Er81 or Runx3, proprioceptive Ia afferents exhibit dramatic but strikingly similar phenotypes at late stages of differentiation suggesting a possible genetic interaction between these two factors with respect to Ia afferent specification.
Nevertheless, the possibility that intrinsic ETS or Runt specific intramolecular modules or properties within either of these proteins could be essential and of predominant functional importance for proper activation of the late Ia afferent transcriptional program, has not been addressed so far. In effect, despite the different highly homologues Pea3 subfamily members of ETS domain proteins, Pea3, ER81 and Erm, appear to harbor some specialized intramolecular properties, they all bind to the same Ets DNA-binding site and share several potential target genes *in vitro* (de Launoit et al., 1997; Sharrocks, 2001; Oikawa and Yamada, 2003). It is however not clear whether the individual subfamily members function in a similar biological manner and might display functional redundancy or in effect functional exclusivity *in vivo*.

This chapter represents an extension of the *in vivo* structure-function analysis of the Pea3 subfamily of highly homologous ETS transcription factors that was described in part in chapter six. Additional *ETS* knock-in mutants were generated allowing a broader analysis of the respective specificity of intramolecular properties within individual Pea3 subfamily members in the assembly of the monosynaptic reflex circuit. Besides, evidence is provided that might suggest that expression of the transcriptional highly active Pea3 variant EWS-Pea3, in the place of Er81, leads to a striking rescue but could not be sufficient to rescue all different aspects of the $Er81^{-/-}$ phenotype to a similar degree. Furthermore, it was found that maintenance of expression of the transcription factor Runx3 in proprioceptive sensory neurons correlates with the degree of rescue of the Ia afferent central projection phenotype in different *ETS* knock-in mice. This correlation might be of functional importance since ETS and Runx transcription factors could synergistically activate target genes that fulfill essential functions in the course of assembly of the monosynaptic reflex circuit.

RESULTS

In Vivo Structure-Function Analysis of the Pea3 Subfamily of ETS Transcription Factors : ETS Knock-in Mutants - Part II

The results that were presented in chapter six provide evidence that Pea3 cannot substitute for Er81 function with respect to the establishment of the ventral Ia afferent termination zone in the spinal cord. In contrast the chimeric fusion protein EWS-Pea3 might harbor the intrinsic capacity to rescue the Er81 mutant phenotype and promote development of proprioceptive Ia afferent projections into the ventral horn of the spinal cord. However, it is not clear to which extent the third member of the Pea3 subfamily of ETS transcription factors, Erm, could compensate for the lack Er81 in $Er81^{-/-}$ mutant mice. To address this issue and to extend the structure-function analysis of the three related ETS proteins Er81, Pea3, and Erm, respectively, further *ETS* knock-in mutant mice were generated. Similar to the targeting strategy described above (chapter six, Figure 27; see also Arber et al., 2000), the coding sequence of mouse *Erm* and of another fusion, *Erm-Pea3*, was introduced into the Er81 genomic locus in frame with the ATG located in the exon 2 using homologous recombination in embryonic stem cells (data not shown). Expression of Er81 was abolished in $Er81^{Erm/-}$ and $Er81^{Erm\text{-}Pea3/-}$ comparable to the findings presented for $ER81^{Pea3/-}$ and $Er81^{EWS\text{-}Pea3/-}$ mutants (Figure 27; data not shown).

Rescue of the Er81$^{-/-}$ Phenotype : Central Ia Afferent Projections

To determine the extent of a potential rescue of Ia proprioceptive afferent projections into the ventral spinal cord axons were traced by immunohistochemistry using antibodies against PV. The approximate degree of rescue of afferent projections into the ventral spinal cord was compared between all *ETS* knock-in mutants (*Er81*$^{NLZ/NLZ}$, *Er81*$^{NLZ/Pea3}$, *Er81*$^{NLZ/Erm}$, *Er81*$^{NLZ/Erm\text{-}Pea3}$, and *Er81*$^{NLZ/EWS\text{-}Pea3}$) and to the control (*Er81*$^{NLZ/+}$). As described in chapter six, significant rescue of arborization of Ia projections was achieved in *Er81*$^{NLZ/EWS\text{-}Pea3}$ mutants (Figures 28D; 56F) whereas only minimal afferent ingrowth into the ventral horn of the spinal cord could be observed in *Er81*$^{NLZ/Pea3}$ mice when compared to *Er81*$^{NLZ/NLZ}$ (Figures 28B, C; 56B; C) and *Er81*$^{NLZ/+}$ (Figures 28A; 56A). *Er81*$^{NLZ/Erm}$ (Figures 56D) and *Er81*$^{NLZ/Erm\text{-}Pea3}$ (Figures 56F) displayed projection phenotypes that were similar to *Er81*$^{NLZ/Pea3}$ (Figures 28C; 56C). At this level of resolution however a clear statement with respect to the exact quantitative extent of the differential rescue observed in the various *ETS* knock-in mutants could not be made. Nevertheless, the findings presented allow the notice that in the absence of Er81 different native and fusion ETS proteins harbor the intrinsic capacity to rescue the Ia afferent projection phenotype to variable degrees with EWS-Pea3 being able to rescue the ingrowth and arborization of Ia afferents in the absence of Er81 to a level comparable to wild-type.

Rescue of the Er81$^{-/-}$ Phenotype : Muscle Spindle Maintenance

In addition to failure of Ia afferents to project to ventrally located motor neurons in the spinal cord in mice lacking Er81, maintenance of peripheral muscle spindles in subsets of limb muscles is severely affected despite initial induction of muscle spindles occurs normally (Arber et al., 2000). Although it is not clear whether the defect in muscle spindle maintenance in *Er81*$^{-/-}$ mutants is a primary spindle intrinsic phenotype (since Er81 is prominently expressed in these structures) or just reflects a secondary effect due to the inappropriate differentiation of the Ia afferents, this muscle spindle phenotype provides a further assay to test the intrinsic potential and specificity of different Pea3 subfamily members with respect to the 'global' assembly of the monosynaptic reflex circuit. In the *gluteus maximus* hind limb muscle roughly 20 intrafusal muscle fibers are embedded within thousands of extrafusal muscle fibers in wild-type animals. These muscle spindles could be visualized by expression of LacZ in *Er81*$^{NLZ/+}$ mice (n>10; Figure 57A; 58H). A complete loss of all muscle spindles in the *gluteus maximus* muscle is observed in absence of Er81 (Figure 57B, see also Arber et al., 2000). Replacement of Er81 by Pea3 (Figure 57C), Erm (Figure 57D) or Erm-Pea3 (Figure 57E) led to minimal, slightly variable, though significant rescue of muscle spindle

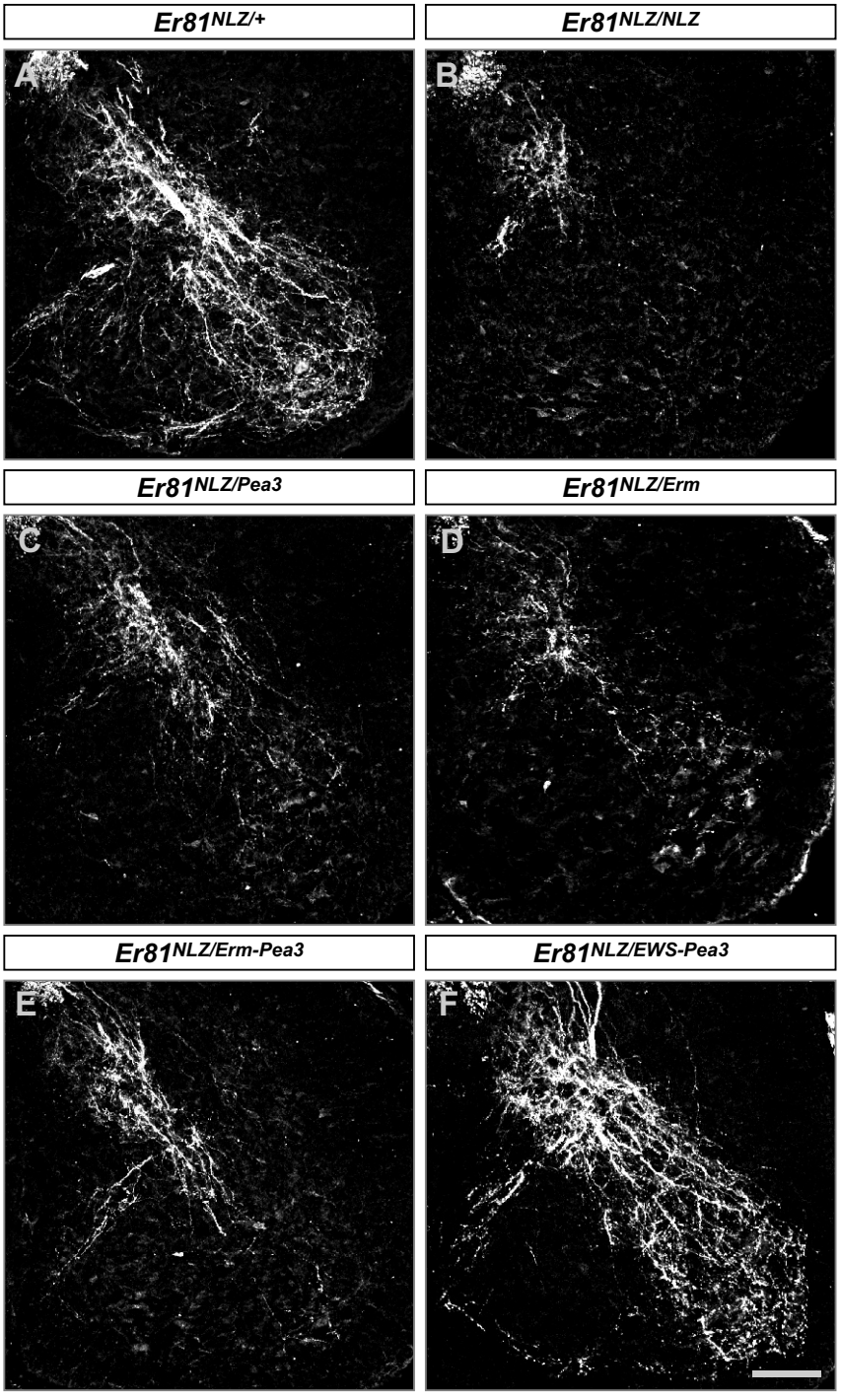


Figure 56. Central Projections of Proprioceptive Afferents in *ETS* Knock-in Mutants. Central projections of proprioceptive afferents in P0.5 lumbar spinal cord of *Er81*$^{NLZ/+}$ (A), *Er81*$^{NLZ/NLZ}$ (B), *Er81*$^{NLZ/Pea3}$ (C), *Er81*$^{NLZ/Erm}$ (D), *Er81*$^{NLZ/Erm\text{-}Pea3}$ (E) and *Er81*$^{NLZ/EWS\text{-}Pea3}$ (F) mice were traced by expression of PV. Scale bar = 150 m.

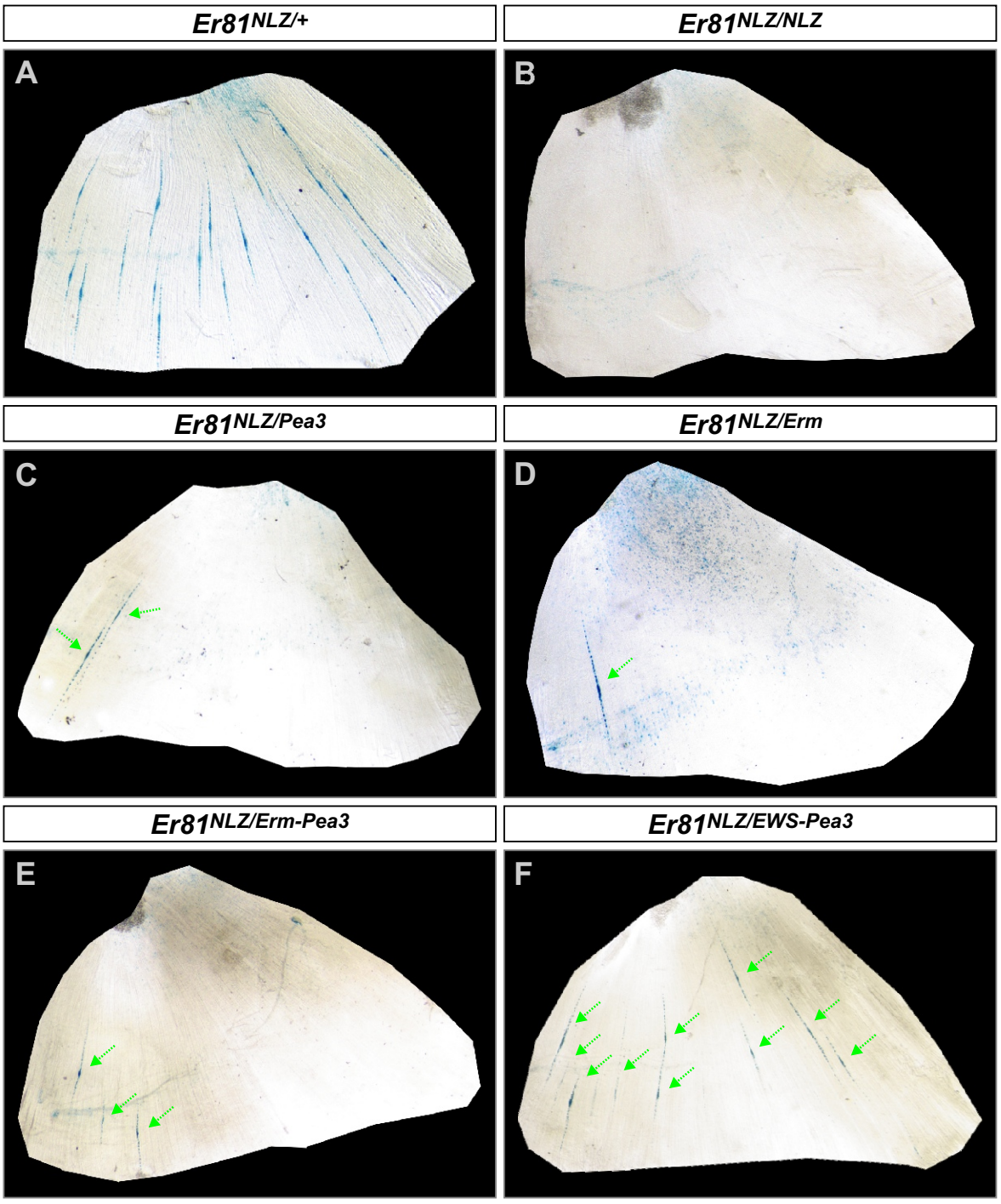


Figure 57. Muscle Spindles in Hindlimb Muscles of *ETS* Knock-in Mutants. Dorsal view of whole-mount LacZ (Gal activity) staining of the *gluteus maximus* muscle of $Er81^{NLZ/+}$ (A), $Er81^{NLZ/NLZ}$ (B), $Er81^{NLZ/Pea3}$ (C), $Er81^{NLZ/Erm}$ (D), $Er81^{NLZ/Erm\text{-}Pea3}$ (E) and $Er81^{NLZ/EWS\text{-}Pea3}$ (F) mice at P7. Green arrows indicate muscle spindles in *ETS* knock-in mutants.

maintenance in the *gluteus maximus* hindlimb muscle. In contrast, in *Er81*$^{NLZ/EWS\text{-}Pea3}$ mutant mice about half the number of the wild-type complement of muscle spindles was detected in each *gluteus maximus* muscle analyzed (n>7; Figures 57F; 58H).

The observed *Er81*$^{NLZ/EWS\text{-}Pea3}$ muscle spindle phenotype is reminiscent to the one reported for *NT3*$^{LacZ/+}$ mice, heterozygous for the neurotrophin NT-3, with respect to the observed number (~50% of wild-type complement) of muscle spindles in the *gluteus maximus* (Figure 58A, B). However, in *NT3*$^{LacZ/+}$ animals at least 50% of proprioceptive Ia afferents are lost due to apoptosis caused by unavailability of sufficient peripheral NT-3 neurotrophic support, providing an explanation for the 50% reduction of the total muscle spindle complement (Farinas et al., 1994; Farinas 1999). Reiterated quantification of PV positive cells in DRG confirmed the loss of proprioceptive DRG neurons in *NT3*$^{LacZ/+}$ in comparison to *NT3*$^{+/+}$ (Figure 58E, G, H). As a consequence, the degree of PV positive axons that enter the dorsal horn and project to ventrally located motor neurons in the spinal cord was significantly reduced in *NT3*$^{LacZ/+}$ heterozygous animals (Figure 58C, D). The equal amount of muscle spindles (~50% of the wild-type complement) present in both *Er81*$^{NLZ/EWS\text{-}Pea3}$ and *NT-3*$^{LacZ/+}$ might suggest that the number of proprioceptive neurons in DRG could also be decreased in a similar range in both *Er81*$^{NLZ/EWS\text{-}Pea3}$ and *NT3*$^{LacZ/+}$ mice. Quantification of PV^{+} cell bodies in DRG of *ER81*$^{NLZ/EWS\text{-}PEA3}$ mutants revealed however no significant decrease in the number of proprioceptive neurons in DRG at E16.5 and P0.5 in comparison to *Er81*$^{NLZ/+}$ (Figure 58E, F, H; data not shown).

Taken together, *NT3*$^{LacZ/+}$ displayed a ~50% reduction in the number of muscle spindles in the *gluteus maximus* because ~50% of Ia afferents were lost. In contrast, *Er81*$^{EWS\text{-}Pea3/-}$ mutant mice failed to maintain ~50% of muscle spindles in the *gluteus maximus* muscle despite a 100% complement of proprioceptive DRG neurons that project into the spinal cord in a wild-type like fashion (Figures 56; 58).

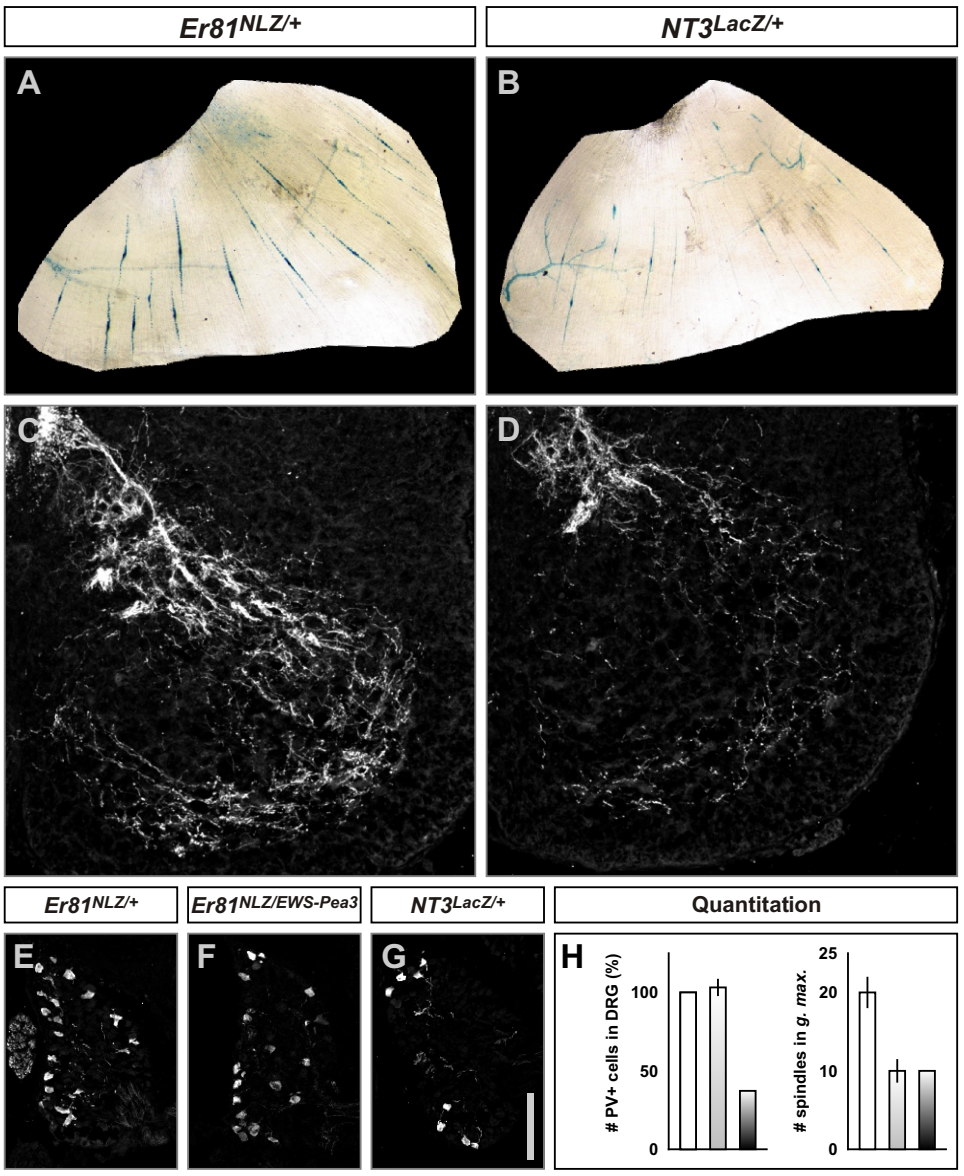


Figure 58. Quantitative Analysis of Proprioceptive Afferents and Muscle Spindles in *NT3*$^{LacZ/+}$ and *Er81*$^{NLZ/EWS\text{-}Pea3}$ mice. (A, B) Dorsal view of whole-mount LacZ (Gal activity) staining of the *gluteus maximus* muscle of *Er81*$^{NLZ/+}$ (A), and *NT3*$^{LacZ/+}$ (B) mice at P7. Note ~50% reduction of Gal-labeled muscle spindles in *NT3*$^{LacZ/+}$ (B) in comparison to *Er81*$^{NLZ/+}$ (A) heterozygous mice. (C, D) Central projections of proprioceptive afferents in P0.5 lumbar spinal cord of *Er81*$^{NLZ/+}$ (C) and *NT3*$^{LacZ/+}$ (D) mice were traced by expression of PV. (E-G) PV expression in lumbar P0.5 DRG of *Er81*$^{NLZ/+}$ (E), *Er81*$^{NLZ/EWS\text{-}Pea3}$ (F) and *NT3*$^{LacZ/+}$ (G) embryos. (H) Quantitation of percentage of proprioceptive PV^{+} afferents in DRG (left chart) and number of muscle spindles in *gluteus maximus* (*g. max*; right chart) in *Er81*$^{NLZ/+}$ (blank columns), *Er81*$^{NLZ/EWS\text{-}Pea3}$ (grey faded columns) and *NT3*$^{LacZ/+}$ (black faded columns) embryos. Significance: percentage of proprioceptive PV^{+} afferents in DRG: *Er81*$^{NLZ/+}$: n>3; *Er81*$^{NLZ/EWS\text{-}Pea3}$: n=2; *NT3*$^{LacZ/+}$: n=1; numbers of muscle spindles in *gluteus maximus*: *Er81*$^{NLZ/+}$: n>7; *Er81*$^{NLZ/EWS\text{-}Pea3}$: n=7; *NT3*$^{LacZ/+}$: n=1. Scale bar: (C, D) = 150 m; (E-G) = 80 m.

Expression of Runx3 in Proprioceptive Afferents in ETS Knock-in Mutants

The similarity in the phenotype with respect to differentiation of proprioceptive afferents in *Er81*$^{-/-}$ and *Runx3*$^{-/-}$ mutant mice (Arber et al., 2000; Inoue et al., 2002) points towards the possibility that Er81 and Runx3 might share similar molecular signaling pathways and/or define part of a genetic cascade responsible for appropriate development of proprioceptive Ia afferent sensory neurons. To begin to address this issue, initial focus was put on the analysis of Runx3 expression in proprioceptive neurons in *Er81*$^{-/-}$ mutants to get a measure whether Runx3 might act genetically downstream of Er81. Runx3 expression in the DRG was analyzed qualitatively using immunohistochemistry (Figure 59). In addition, the mean number of Runx3 expressing neurons in the DRG was determined using a consistent quantification approach (Figure 60; see Experimental Procedures for details). In *Er81*$^{NLZ/+}$ heterozygous embryos at E16.5, Runx3 was prominently expressed in ~12 cells in lumbar DRG/section (Figure 59A-C) whereas in *Er81*$^{NLZ/NLZ}$ mutant embryos Runx3 was only expressed in ~5 nuclei in the DRG (Figure 59D-F). Similar quantitative results were obtained when expression of Runx3 was followed in Er81 positive cells (monitored by LacZ expression). Whereas in *Er81*$^{NLZ/+}$ embryos ~10 cells coexpressed Runx3 and LacZ *Er81*$^{NLZ/NLZ}$ embryos displayed ~3 yellow cells in lumbar DRG. This result might suggest that expression of Runx3 could be under direct or indirect control of Er81 in a fraction of up to 60-70% of proprioceptive DRG neurons. Furthermore, this aspect of the *Er81*$^{-/-}$ phenotype provides another clear and efficient assay to analyze the potential of individual Pea3 subfamily members to compensate for the lack of Er81. Quantification of the number of Runx3 positive cells in lumbar DRG of the different ETS knock-in mutants revealed that in *Er81*$^{NLZ/EWS\text{-}Pea3}$ mutant DRG Runx3 expression could be detected in ~12 cells similar to wild-type (Figure 59G-I). In contrast, *Er81*$^{NLZ/Erm}$ and *Er81*$^{NLZ/Erm\text{-}Pea3}$ displayed a marked reduction of Runx3 expression more in the quantitative region of the *Er81*$^{NLZ/NLZ}$ mutant (Figure 59D-F, J-O). To determine the significance of these findings the numbers of counts of a minimum of 50 sections/genotype were partially pooled and displayed in comparative charts (Figure 60). In summary, these findings indicate that the degree of rescue of Ia afferents establishing a ventral termination zone in the spinal cord directly correlates with the number of proprioceptive sensory neurons maintaining expression of the transcription factor Runx3.

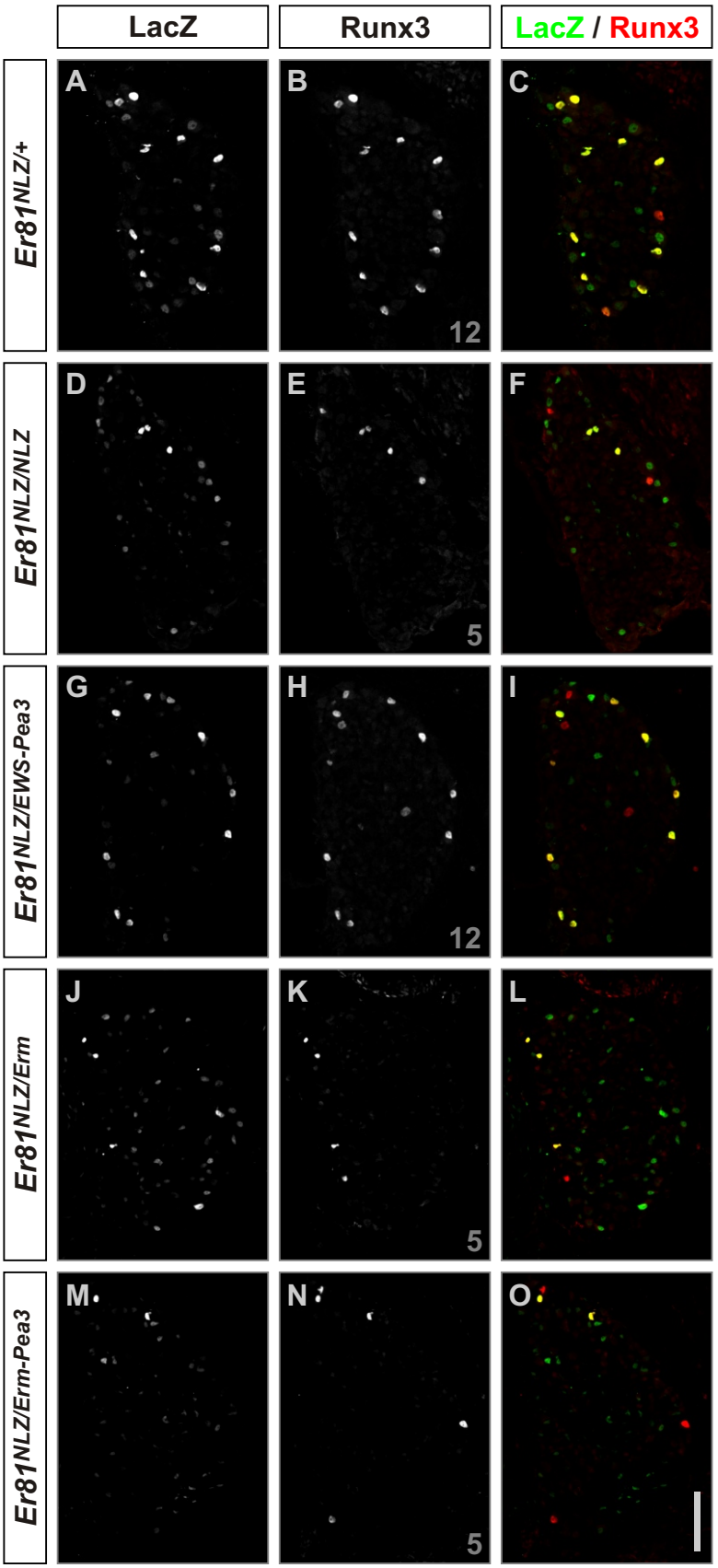


Figure 59. Expression of Runx3 in DRG of *ETS* Knock-in Mutants. Analysis of LacZ (white: A, D, G, J, M; green: C, F, I, L, O) and Runx3 (white: B, E, H, K, N; red: C, F, I, L, O) expression in lumbar DRG in of *Er81*$^{NLZ/+}$ (A-C), *Er81*$^{NLZ/NLZ}$ (D-F), *Er81*$^{NLZ/EWS-Pea3}$ (G-I), *Er81*$^{NLZ/Erm}$ (J-L), *Er81*$^{NLZ/Erm-Pea3}$ (M-O) animals at E16.5 (A-I) and P0.5 (J-O). Scale bar: (A-I) = 70 m; (J-O) = 80 m.

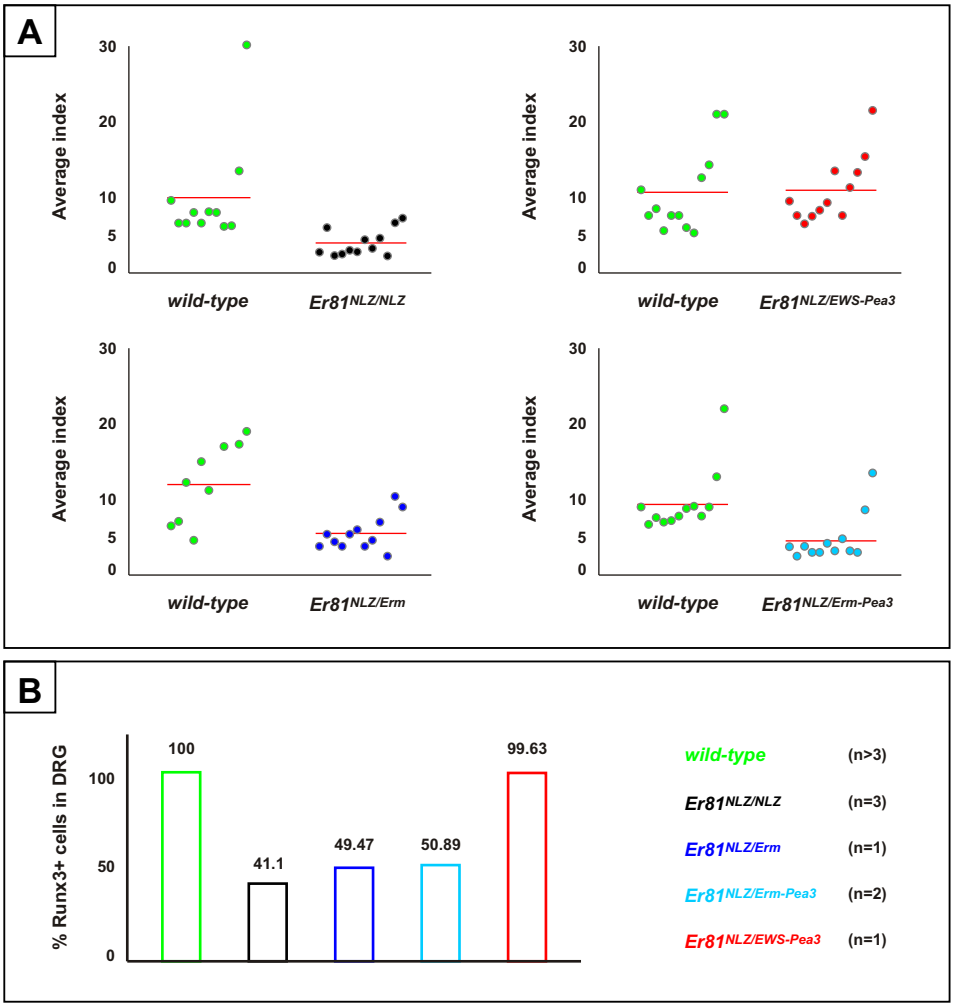


Figure 60. Quantitative Analysis of Runx3 Expression in DRG of *ETS* Knock-in Mutants. (A) Average indices (for details see Experimental Procedures) obtained from counts of Runx3^{+} DRG neurons in *Er81*$^{NLZ/NLZ}$ (top left; black), *Er81*$^{NLZ/EWS-Pea3}$ (top right; red), *Er81*$^{NLZ/Erm}$ (bottom left; blue), *Er81*$^{NLZ/Erm-Pea3}$ (bottom right; cyan) animals were each plotted in an individual chart together with average indices obtained from a corresponding wild-type (green) littermate. The average of mean of all data points/genotype within the respective chart is indicated (red line). (B) Quantitation of percentage (pooled average indices) of Runx3^{+} neurons in DRG in different *ETS* knock-in mutants summarized in a comparative chart.

DISCUSSION

The experimental data presented in this chapter provide a piece of an *in vivo* structure-function analysis of the Pea3 subfamily of ETS transcription factors. Functional specificity with respect to a defined biological role of different members, Pea3, Er81, and Erm was addressed in the context of neuronal differentiation and the assembly of the monosynaptic reflex circuit within the developing murine spinal cord. It was found that different Ets proteins Pea3, Er81 and Erm appear to fulfill probably nonredundant functions with respect to establishment of the Ia afferent termination zone in the ventral horn of the spinal cord. Nevertheless, the chimeric transcriptionally highly active EWS-Pea3 fusion protein harbors an intrinsic molecular potential that allows partly to substitute for Er81 function within proprioceptive afferents. However, not every aspect of the $Er81^{-/-}$ mutant phenotype could be rescued to the same extent in $Er81^{EWS\text{-}Pea3/-}$ mice indicating that subtle changes in transcriptional specificity in EWS-Pea3 compared to Er81 might account for these phenotypic differences. The following discussion will focus on the molecular *in vivo* specificity of the Pea3 subfamily with respect to differentiation of proprioceptive Ia afferents and the assembly of the functional monosynaptic reflex circuit.

Specificity in Sensory-Motor Connectivity : Pea3 Subfamily

Proprioceptive Ia afferent DRG sensory neurons require specific molecular cell intrinsic properties besides extracellular factors that are encountered by Ia afferent axons and cell bodies. More specifically, expression of the ETS transcription factor Er81 represents a critical cell intrinsic modulator of the Ia afferent differentiation program. In absence of Er81, Ia afferents fail to establish the ventral termination zone where direct monosynaptic synapses would form on dendrites of α-motor neurons (Arber et al., 2000). As a consequence, $Er81^{-/-}$ mutant animals are highly ataxic and show uncoordinated movements of their limbs due to the absence of synaptic inputs from the sensory unit to the motor unit as a result of the disruption of the reflex circuit (Arber et al., 2000). However, the exact role(s) of Er81 in Ia afferents in the processes of ventral arborization and branching, the formation of synaptic specializations on dendrites and establishment of specific selective synapses is still an open issue. Nevertheless, the possibility that Er81 fulfills essential tasks in all of these processes cannot be excluded. Future experiments using conditional mouse genetic approaches that allow the elimination of *Er81* expression at late stages, when the Ia afferent termination zone in the ventral spinal cord has been established already, might resolve whether Er81 function is indeed required to fulfill additional tasks (besides the initial promotion of ventral

Ia afferent arborization), such as in situ induction of synaptic specializations or more importantly the granting of synaptic specificity and selectivity that is achieved in sensory-motor connectivity.

While it is clear, that Er81 cannot be replaced by the highly homologous subfamily members Pea3 or Erm it appears that the 'artificial' EWS-Pea3 fusion can promote the establishment of ventral projections of Ia afferents in the absence of Er81. In terms of molecular specificity several interesting points can be made.

The ETS DNA binding domain might be interchangeable with respect to the promotion of Ia afferent projections to the ventral spinal cord (at least for Pea3 and Er81). All three, the Pea3, Er81, and Erm ETS domains have been shown to bind to the same ETS DNA-binding site *in vitro* (Brown and McKnight, 1992; Monte et al., 1994; Brown et al., 1998). Therefore, it is reasonable to suggest that a significant ETS specific subset of target genes, required for the process of Ia ventral arborization, are activated by Er81 as well as by the EWS-Pea3 fusion. However, this would imply that native Pea3 and Erm activity is too low to achieve the same effect. In contrast, differences in specific intra- and intermolecular properties might account for the inability of Pea3 and Erm to act in a functionally redundant way like Er81 or EWS-Pea3. In particular, intra-molecular autoinhibitory modules that are not present in Er81 (and probably not anymore in EWS-Pea3) could block Pea3 and Erm activity (Greenall et al., 2001, Bojovic and Hassell, 2001). Furthermore, absence of appropriate triggers or cofactors in Ia afferents to relieve this autoinhibition might explain reduced activation of Pea3 and Erm whereas the structural conformation of EWS-Pea3 could disturb the appropriate folding of inhibitory modules and therefore render EWS-PEA3 constitutively active and/or independent of any signaling stimuli or cofactors.

The intrinsic transactivation potentials of both Pea3 and Erm are significantly lower *in vitro* (T. Portmann, S. Hippenmeyer and S. Arber, unpublished observation) than that of EWS-Pea3 and could provide an explanation for lowered capability to activate gene expression *in vivo*. Interestingly, isolated cell culture transcriptional assays however revealed that the intrinsic transactivation potential of Er81 is even lower than the potentials observed in Pea3 or Erm (T. Portmann, S. Hippenmeyer and S. Arber, unpublished observation). These findings suggest that if the transactivation potential is a critical functional *in vivo* parameter, Er81 essentially requires activation through signaling input and/or intermolecular interactions with appropriate partner molecules. These activation mechanisms might lead to substantial Er81 specific posttranslational modifications that in turn would render Er81 sufficiently active but not Pea3 or Erm (Janknecht, 1996; Baert et al., 2002; Goel and Janknecht, 2003).

Specificity in Sensory-Motor Connectivity : ETS and Runt

Runx3 might represent a potential candidate co-regulatory synergistic partner for Er81 and/or Erm but probably not Pea3 as transcriptional assays provided evidence that Er81 and Erm but not Pea3 might synergize with Runx3 to transactivate and induce expression of luciferase from a reporter plasmid (T. Portmann, S. Hippenmeyer, I. Kramer and S. Arber, unpublished observation). In addition, Runx3 appears to represent one of the first common downstream effectors of both Er81 and interestingly also EWS-Pea3. It is however not clear how direct Er81 and EWS-Pea3 control maintenance of expression of Runx3 in Ia afferents. Nevertheless, Runx3 activity alone in the absence of Er81 might probably not substitute for the lack of Er81 (I. Kramer and S. Arber, personal communication) and therefore could not be the only and essential Er81 downstream effector. In the light of a possible synergistic interaction between Er81 and Runx3 it appears reasonable that expression of both would be required to achieve induction of the appropriate downstream transcriptional program. To test this synergistic activation hypothesis *in vivo* in the context of Ia afferent differentiation is however not trivial since expression of Runx3 seems to be controlled by Er81. Nevertheless, Erm appears to synergize as well with Runx3 in transcriptional activation assays and binds to identical ETS DNA-binding sites like Er81 *in vitro* (Brown and McKnight, 1992; Monte et al., 1994). Therefore, if a high ETS specific transactivation potential would be a crucial parameter to promote establishment of a ventral Ia afferent termination zone irrespective of the DNA domain present, rescue of Runx3 levels in Ia afferents in $Er81^{Erm/-}$ or $Er81^{Erm\text{-}Pea3/-}$ mice by conditional expression, using a genetic strategy to bypass expression from the endogenous *Runx3* genomic locus, should allow to address this hypothesis.

Specificity in Sensory-Motor Connectivity : Er81 vs. EWS-Pea3

Expression of *EWS-Pea3* from the endogenous *Er81* genomic locus in an $Er81^{-/-}$ background leads to morphological rescue of the Ia afferent termination zone in the ventral horn of the spinal cord according to visual evaluation of the arborization pattern of either PV^{+} or dextran filled axonal projections. Furthermore, physiologically functional synapses with α-motor neurons are formed, although in mice expressing *EWS-Pea3*, instead of Er81, only ~60% of the electrical input can be recorded in extracellular recordings. These findings indicate that either not 100% of all synapses might be present and/or that synaptic strength of individual synapses could be reduced in $Er81^{EWS\text{-}Pea3/-}$ mice (E. Vrieseling, D.R. Ladle, S. Hippenmeyer and S. Arber, unpublished observation). Alternatively, reduced amounts of available peripheral supplements such as neurotrophins, that have been shown to maintain the strength of synaptic connectivity between Ia afferents and motor

neurons in the ventral spinal cord (Mendell, 2001; Chen et al., 2003), might account for reduced synaptic transmission in *Er81*$^{EWS\text{-}Pea3/-}$ mice. In particular, the neurotrophin NT-3 synthesized by intrafusal muscle spindles appears to be required from early postnatal stages on for maintenance of normal excitatory postsynaptic potentials (EPSPs) that Ia afferents evoke in motor neurons (Chen et al., 2002b). Mutant mice with a targeted genomic deletion of the zinc-finger transcription factor *Egr3* fail to maintain muscle spindles in all skeletal muscles (Tourtelotte and Milbrandt, 1998). However, initial induction and differentiation of muscle spindles appeared normal in newly born *Egr3*$^{-/-}$ mice as assessed by expression of *Pea3*, *Erm*, *Er81* and *NT-3*, and the morphology of Ia afferent terminals (S. Hippenmeyer and S. Arber, unpublished observation). Nevertheless, intrafusal muscle fibers progressively degenerate within the first two postnatal weeks (Tourtelotte et al., 2001) and therefore muscle spindle derived NT-3 is lost in mice lacking Egr3. Interestingly, synaptic connections between Ia afferents and motor neurons are already established and the ventral Ia afferent termination zone in the spinal cord is maintained in *Egr3*$^{-/-}$ mice at the time of muscle spindle degeneration. Strikingly, motor neuron EPSPs are small in the absence of endogenous muscle spindle derived NT-3 but can be restored through repetitive intramuscular injections of NT-3 in *Egr3*$^{-/-}$ mice (Chen et al., 2002b).

The failure of muscle spindle maintenance in subsets of hind limb muscles has been shown for *Er81*$^{-/-}$ mice (Arber et al., 2000). Similar to *Egr3*$^{-/-}$ mutant mice, muscle spindle induction and early differentiation occurs normal and intrafusal muscle fibers are lost only after E18.5 within the first postnatal week (Arber et al., 2000). However, rescue of this aspect of the *Er81*$^{-/-}$ phenotype is not complete in *Er81*$^{EWS\text{-}Pea3/-}$ animals. Rather, several hind limb muscles such as *gluteus maximus* maintain only ~50% instead of the full wild-type complement of muscle spindles. Furthermore, recent evidence might suggest that expression of NT-3 could be controlled through Er81 in muscle spindles (S. Hippenmeyer and S. Arber, unpublished observation) and whether EWS-Pea3 might restore intrafusal NT-3 expression to wild-type levels in *Er81*$^{EWS\text{-}Pea3/-}$ mutant hind limb muscles remains to be shown.

In conclusion, the number of muscle spindles and therefore the amount of intrafusal NT-3 might crucially account for maintenance of synaptic strength between Ia afferents and α-motor neurons. Therefore, the observed reduction of synaptic transmission in *Er81*$^{EWS\text{-}Pea3/-}$ mutants might represent a reflection of the reduced complement of muscle spindles in subsets of hind limb muscles due to incomplete rescue by EWS-Pea3.

A comparison of the behavioral phenotype of *Er81*$^{EWS\text{-}Pea3/-}$ in comparison to *Er81*$^{-/-}$ or wild-type animals revealed that despite formation of functional synapses these animals still displayed severe ataxia similar to *Er81*$^{-/-}$ mice (data not shown; Arber et al., 2000). Interestingly, *NT-3*$^{LacZ/+}$ heterozygous mice lacked more than 50% of proprioceptive sensory neurons in DRG and showed only ~40% of active synaptic transmission from Ia afferents to motor neurons but apparently displayed normal motor behavior suggesting that the absolute number of Ia afferent axonal projections in the ventral spinal cord might not be very critical for proprioception (S. Hippenmeyer, E. Vrieseling and S. Arber, unpublished observation). Further, these findings point towards a direct correlation between quantitative synaptic Ia afferent - motor neuron transmission and the number of proprioceptive sensory neurons present in DRG, and rise the question why *Er81*$^{EWS\text{-}Pea3/-}$ mice show ataxia but display the wild-type complement of proprioceptive DRG neurons, extensive arborization into the ventral spinal cord and significant synaptic transmission to motor neurons? A reasonable causal basis for these observations includes the possibility that Ia afferents might loose an intrinsic specificity control mechanism and could form inappropriate and/or ectopic synapses on dendrites of incorrect α-motor neurons. This lack of selectivity could in turn result in spatially and temporally discoordinated EPSPs and/or inappropriate action potentials within α-motor neurons in response to Ia afferent input. Indeed, preliminary results obtained from intracellular recording experiments in *Er81*$^{EWS\text{-}Pea3/-}$ mutant animals provide evidence that Ia afferents not only form correct synapses on homonymous but also inappropriate ectopic but functional synaptic contacts on dendrites of antagonistic α-motor neurons (DR. Ladle, E. Vrieseling, S. Hippenmeyer and S. Arber, unpublished observation). It will be interesting to determine the exact degree of morphological rescue of axonal Ia afferent ventral projections in a correlation to the precise evaluation of the actual number of physiologically functional synapses formed on homonymous compared to antagonistic α-motor neuron dendrites in *Er81*$^{EWS\text{-}Pea3/-}$ mice.

The issues discussed above clearly point towards differences in the molecular action of EWS-Pea3 in comparison to Er81. Although the given likelihood that a substantial subset of Er81 target genes is activated by EWS-Pea3, differences with respect to some aspects of the *Er81*$^{EWS\text{-}Pea3/-}$ phenotype when compared to wild-type might suggest significant changes in the Ia afferent transcriptional profile that is generated by Er81 or EWS-Pea3, respectively. These differences might include Er81 specific target genes that are not induced by EWS-Pea3 and conversely a subset of genes could be upregulated exclusively by EWS-Pea3 but not Er81. In effect, it is generally believed that the exchange of the endogenous ETS transcriptional transactivation domains with the EWS NTD generates EWS-ETS chimeric transcription factors with some novel biochemical and genetic *in*

vivo specificities despite the fact that EWS-ETS bind to DNA in a site-specific manner *in vitro* (Kim and Pelletier, 1999; de Alava and Gerald, 2000; Arvand and Denny, 2001). As already mentioned, changes in the interactions with co-regulatory partner proteins or intramolecular transactivation properties might account for these novel transcriptional signaling specificities. Nevertheless, the possibility that the Pea3 and Er81 ETS domain are not 100% interchangeable with respect to the selection of Er81 specific target genes cannot be excluded completely. Structural analyses have revealed that even highly homologous subfamily members of ETS proteins might display slightly different, very subtle DNA-binding preferences with respect to related or even identical DNA targets despite strict conservation of the ETS domain residues that contact the DNA (Shore and Sharrocks 1995; Shore et al., 1996) Moreover, DNA binding specificity determinants appear to include not only the conserved ETS domain but in addition non-conserved residues located in less homologous DNA-distal domains within distinct ETS subfamily members (Mo et al., 1998; Mo et al., 2000).

In summary, while expression of EWS-Pea3 in an *Er81* mutant context promotes both morphological and functional rescue of the *Er81*$^{-/-}$ phenotype to a strikingly high degree, some aspects with respect to the complete and physiologically functional assembly of the monosynaptic spinal reflex circuit appear to be supported exclusively by the ETS transcription factor Er81. Furthermore, EWS-Pea3 might harbor the capability to actively promote synaptogenesis at inappropriate ectopic dendritic target sites on antagonistic α-motor neurons. It will be interesting to resolve the changes in the transcriptional programs induced by Er81 versus EWS-Pea3 as this approach could lead to the identification of endogenous Ia afferent ‘selective synapse promoting’ molecules that ensure appropriate hard-wiring of the monosynaptic stretch reflex circuit.

Chapter 9

Open Issues

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Perspectives

Assembly of the Monosynaptic Stretch Reflex Circuit - Intrinsic and Extrinsic Signals on Time

Assembly of the spinal monosynaptic stretch reflex circuit depends on distinct genetic programs initiated at successive phases during development. Modulation and regulation of these genetic programs is mediated through several classes of transcription factors. A significant body of information has been acquired with respect to transcriptional mechanisms involved in the specification of progenitor cell populations of motor neurons in the spinal cord (Jessell, 2000; Shirasaki and Pfaff, 2002) and the process of initial generation of DRG neurons (Anderson et al., 1997; Anderson, 2000; Bertrand et al., 2002; Knecht and Bronner-Fraser, 2002). However, the molecular signaling cascades and transcriptional regulators that are involved in postmitotic differentiation and specification of both motor and DRG sensory neurons are only beginning to be revealed. One recently emerging concept involves the regulation of terminal specification through peripheral target derived factors that signal retrogradely to shape the differentiation program of Ia afferents as well as motor neurons by modulation of specific transcriptional programs. More specifically, distinct peripheral signals, GDNF and NT-3, regulate expression of two members of the Pea3 subfamily of ETS transcription factors, Pea3 and Er81, which in turn fulfill essential roles in late differentiation steps of both, motor and Ia afferent sensory neurons (Arber et al., 2000; Livet et al., 2002; Haase et al., 2002; Patel et al., 2003).

A crucial aspect of ETS transcriptional signaling in Ia afferents and motor neurons appears to include a tight temporal regulation of the onset of ETS transcriptional activity (chapters six and seven within this thesis). Molecules that are functionally coupled to the selection of the appropriate temporal window however remain to be identified. It is likely that the dynamic state and conformation of chromatin during specific phases of specification and differentiation allows or inhibits the expression of appropriate genetic programs (Müller and Leutz, 2001; Kouzarides, 2002). Furthermore, transcriptional repression mechanisms have been shown to be involved in the specification of motor neuron progenitor cell populations (Muhr et al., 2001). Such repression mechanisms might block the access to promoters and enhancers in a conceptually similar manner also in postmitotic neurons and thereby prevent transcriptional activators from activating gene expression during inappropriate time windows. Signaling cascades that both regulate temporal chromatin remodeling or relieve of transcriptional repression might modulate the competence of α-motor neurons and/or Ia afferents to activate distinct transcriptional programs during defined temporal windows and thus could contribute to the timely appropriate specification of Ia afferents and/or α-motor neurons during assembly of the monosynaptic reflex circuit in the spinal cord.

Peripheral Specification of Central Connectivity

What are the molecular determinants responsible for promotion of central Ia afferent projections and/or the formation of selective synaptic connections of Ia afferents with α-motor neurons? In effect, there are only a handful of potential candidate molecules known to be responsible for, or involved in the assembly of the monosynaptic reflex circuit in the spinal cord (Chen et al., 2003). Nevertheless, the actual molecular signaling pathways controlling selectivity in the formation of specific synaptic contacts between particular Ia afferents and the corresponding homonymous α-motor neurons are unknown.

The formation of specific Ia afferent central connections to α-motor neurons occurs mostly independent of patterned neuronal activity (Mendelson and Frank, 1991) but involves environmental specification through essential target derived peripheral signals. In particular, if Ia afferents normally projecting into ventral limb muscles are forced to supply a duplicate set of dorsal limb muscles in chicken embryos they form functional connections specifically with dorsal motor neurons that innervate the corresponding normal dorsal muscle (Frank and Wenner, 1993; Wenner and Frank, 1995). The exact nature of these peripheral signals controlling the appropriate motor neuron selection by Ia afferents in the ventral spinal cord is however currently unknown.

In chick the coordinate expression of Pea3 and Er81 by both motor neurons and corresponding presynaptic Ia afferents might be of functional relevance for establishment of selective monosynaptic connectivity since motor neurons and proprioceptive afferents innervating the same muscle express the same ETS transcription factor (Lin et al., 1998).
The entire process of selectivity of synaptic connectivity between Ia afferents and α-motor neurons can however not be explained purely by virtue of ETS gene expression since in the mouse, Er81 is expressed by all proprioceptive sensory neurons (Arber et al., 2000). But if identical ETS gene expression within particular interconnected subpopulations of Ia afferents and α-motor neurons are part of the molecular mechanistic basis of specificity in connectivity in chick embryos, what are the transcriptional downstream target genes that control the formation of specific synaptic connections? Homophilic cell surface interactions might provide means for a selective matching of sensory axons and motor neurons that are part of the same circuit. Several findings suggest that the expression of type II Cadherins and ETS genes might be linked in motor and probably also in sensory neurons. First, it has been shown that both ETS and Cadherin (Cad) expression in motor and/or sensory neurons is dependent on limb-derived signals (Lin et al., 1998; Price et al., 2002). Second, ectopic expression of *Er81* results in the deregulation of motor neuron MN-Cad expression

in the chick spinal cord (Price et al., 2002). Third, the analysis of *Pea3*$^{-/-}$ mutant mice indicates that expression of type II Cadherins, notably Cad-7 and Cad-8, in specific motor pools, within the brachial LMC, is regulated by this ETS gene (Livet et al., 2002).

In summary, the relation between ETS transcription factors and type II Cadherins could provide part of a basis for the selectivity with which monosynaptic connections are formed and future work will hopefully reveal the actual molecular mechanisms by which selective synapses between Ia afferents and α-motor neurons form during development.

A Role for Motor Neurons in Ia Afferent Arborization and/or Formation of Selective Synaptic Connections in the Ventral Spinal Cord ?

The process of establishing a central Ia afferent termination zone includes that Ia afferents arborize extensively and 'fine-branch' once their axons have reached the ventral horn of the spinal cord (Brown, 1981). Thus Ia afferents could potentially form synaptic contacts with many different α-motor neurons innervating distinct muscles. An important step in understanding the process of selective synapse formation between Ia afferents and α-motor neurons therefore is to define molecular regulators responsible for appropriate (fine-) branching of Ia afferent axons within the ventral spinal cord in close proximity to target dendrites. Do Ia afferents branch randomly in their target region with concomitant pruning of inappropriate axonal extensions? Are factors secreted by α-motor neurons of importance in Ia afferent branching processes? Recent evidence suggests that α-motor neurons are not required to attract Ia afferents towards α-motor neurons since even in their absence by selective expression of DTX in early postmitotic motor neurons Ia afferent projections reach the ventral horn of the spinal cord (Patel et al., 2003). Nevertheless, the possibility, that α-motor neurons might secrete 'secondary branching factors' cannot be excluded. It has been shown that Wnt3 is expressed by LMC motor neurons (Krylova et al., 2002). Furthermore, DRG sensory neurons cultured in the presence of Wnt3 display larger growth cones and branch more elaborately (Krylova et al., 2002). However, a direct role for Wnt3 or other local unknown α-motor neuron derived factors in the process Ia afferent branching and selective synapse formation between Ia afferents and α-motor neurons *in vivo* remains to be demonstrated. Moreover, even a role for yet to be identified α-motor neuron intrinsic mechanisms rendering them competent to receive circuit specific input from Ia afferents on particular 'dendritic hotspots' might be considered. Such 'late' fine-tuning of α-motor neuron competence could in addition be under peripheral regulation by retrograde signals originating from the particular muscle that is innervated by the α-motor neurons

and the corresponding Ia afferents and contribute to the specificity with which monosynaptic connections are formed in the spinal cord. The importance of motor neuron intrinsic transcriptional signaling programs in controlling their competence to receive appropriate synaptic input has been demonstrated in *C. elegans* where specific VA motor neurons lacking UNC-4/UNC-37 dependent transcriptional repression, are miswired and receive inappropriate synaptic inputs from interneurons normally destined for lineal sister VB motor neurons (Winnier et al., 1999). Future studies might reveal whether similar molecular cascades involved in shaping the potential of motor neurons to receive specific synaptic input could also be relevant and/or conserved in motor neurons within the murine spinal cord.

ETS Signaling in Ia Afferents and Specificity in Sensory-Motor Connectivity

The ETS transcription factor Er81 is required to promote the establishment of the ventral Ia afferent termination zone in the spinal cord (Arber et al., 2000). Simple studies eliminating Er81 function throughout development have thus not allowed addressing the question whether Er81 could be involved in controlling additional steps in the formation of monosynaptic connections. It will therefore be interesting in future experiments to conditionally eliminate Er81 expression after Ia afferents have established the ventral termination zone to address the question whether Er81 activity contributes to selectivity in sensory-motor connectivity. Interestingly, it is not uncommon, that the same transcriptional regulator is involved in several successive steps of postmitotic neuron differentiation to control specifically the choice of axonal pathway, establishment of branching pattern and the recognition of synaptic targets (Marie et al., 2002).

The *in vivo* exchange of *Er81* with the *EWS-Pea3* fusion leads to extensive arborization in the ventral horn of the spinal cord. Furthermore, synaptic specializations of Ia afferent axons onto α-motor neuron dendrites are formed despite the presence of both specific (homonymous connections) and ectopic unspecific (antagonistic connections) synapses. These findings indicate that EWS-Pea3 has the potential to execute a functionally similar role to Er81. Therefore, EWS-Pea3, although representing a rather 'artificial ETS protein', might regulate a significant subset of Er81 target genes that are required for the extension and branching of Ia afferents into the ventral spinal cord. Importantly, these findings could also suggest that the process of Ia afferent - α-motor neuron synaptogenesis could require indeed the presence of ETS specific transcriptional activity in Ia afferents. Nevertheless, the presence of ectopic inappropriate synapses onto inappropriate dendrites of antagonistic α-motor neurons in $Er81^{EWS\text{-}Pea3}$ mice might reflect the induction of

inappropriate transcriptional events that are exclusive to EWS-Pea3, but not Er81, however mediated via ETS DNA-binding sites.

Tight regulation of specific transcriptional programs involved in the establishment of highly selective synapses onto appropriate targets has also been proposed to play a crucial role for insect mechanosensory neurons. More specifically, particular peripheral hair cell associated sensory neurons (e.g. 6m) in the cockroach larvae project to defined (terminal ganglion) interneurons and form highly selective connections (Marie et al., 2000). Expression of the transcription factor Engrailed during several successive postmitotic stages of differentiation is essential for axon guidance, branching and selective synaptogenesis of the sensory neuron 6m (Marie et al., 2002). Interestingly, knock down of *engrailed* expression at postmitotic stages in 6m results in a change of synaptic connections: The pattern of selective synaptic connections made by 6m that are en^- is altered in such that the strength of normal connections is reduced and new ectopic synapses with inappropriate targets are formed (Marie et al., 2002).

In summary, the absence of expression of a transcription factor (En) at defined developmental stages or the expression of a transcriptional regulator with similar but slightly different molecular properties (EWS-Pea3) instead of the endogenous one (Er81) in defined neuronal subpopulations could result in subtle but significant changes in the transcriptional genetic profile partly incompatible with the formation of selective and specific synaptic connections with appropriate target neurons. With respect to spinal monosynaptic connections, determination of these transcriptional changes evoked by EWS-Pea3 in comparison with Er81 might lead to the identification key candidate downstream targets which fulfill essential roles in the formation of specific selective monosynaptic connections between Ia afferents and α-motor neurons.

Transcriptional Mode of Action of Endogenous and 'Artificial' ETS Proteins

In comparison to native Pea3, EWS-Pea3 evoked cellular Ia afferent responses might have the functional molecular basis in the N-terminal transactivation domain of EWS. The change in the molecular properties (increased transactivation potential, relieve of autoinhibition or a change in requirement for co-regulatory partner proteins) might be one possible explanation for the difference in action of Pea3 and EWS-Pea3 in Ia afferents. However, there is evidence that endogenous native full length *EWS* is also expressed in DRG sensory neurons although not in distinct subpopulations (S. Hippenmeyer and S. Arber, unpublished observation). EWS has been shown to interact *in vitro*

with the transcriptional cofactors p300/CBP (Rossow and Janknecht, 2001) that could represent potential co-regulatory proteins also for Er81 (Papoutsopoulou and Janknecht, 2000). Interestingly, both p300 and CBP have been described to be expressed in subpopulations of DRG sensory neurons (Partanen et al., 1999). Might expression of *EWS-Pea3*, despite being an ETS specific regulator, interfere with endogenous EWS activity that could possibly be linked to processes involved in the assembly of the monosynaptic circuit? Only gene targeting of *EWS* could provide some insight into a putative role of endogenous EWS in the differentiation of Ia afferent sensory neurons.

It is not clear, whether the increased transactivation potential observed for EWS-Pea3 *in vitro* when compared to native Er81 or Pea3 might be the only driving force to upregulate ETS target genes responsible for elaboration of the ventral termination zone in the spinal cord (see also discussion on ETS domain specificity issues within chapter eight). Furthermore, to what extent is EWS-Pea3 capable to activate Er81 target genes? It is not known whether Er81 and Pea3 share common target genes *in vivo* despite extensive sequence homology and the fact that they bind to identical ETS DNA-binding sites *in vitro*. (Laudet et al., 1999; Brown and McKnight, 1992; Brown et al., 1998). It is however interesting to note that different EWS-ETS fusions harbor an almost identical *in vitro* transactivation potential. Specifically, the three EWS-Fli-1, EWS-Er81, and EWS-Pea3 chimeric fusions transactivate the luciferase reporter to very similar levels (T. Portmann, S. Hippenmeyer and S. Arber, unpublished observation). In addition, some target genes are transcriptionally regulated by both fusions EWS-Fli-1 and EWS-Er81, respectively, *in vitro* (Hahm et al., 1997; Im et al., 2000). Fusions with an identical transactivation domain (NTD of EWS) would therefore potentially allow a direct comparison of the specificity of the ETS DNA-binding domain with respect to activation of specific ETS target genes *in vivo*. The fusion of transactivation domains with higher potency than the N-terminal domain of EWS, such as the acidic transactivation domain of the viral protein VP16, to the ETS domain of either Er81 or Pea3 might provide further insight into specific quantitative transactivation requirements for the selection and appropriate induction of expression of ETS target genes *in vivo* when compared to the EWS-ETS fusions or Er81 and Pea3, respectively. It would be interesting to analyze the effects of VP16-ETS fusions *in vivo* with respect to the assembly of the monosynaptic circuit especially as both VP16-Er81 and VP16-Pea3 transactivate the luciferase reporter to significantly higher absolute levels when compared to EWS-Er81 and EWS-Pea3, respectively (T. Portmann, S. Hippenmeyer, C. Laengle and S. Arber, unpublished observation). An in-depth cross-comparison of the molecular properties of the different EWS-ETS, VP16-ETS and ETS-ETS fusion proteins might contribute to the evaluation of particular intramolecular requirements. Specifically, such a study might reveal molecular features

of the transactivation and the ETS DNA-binding domain of different Pea3 subfamily members of ETS transcription factors required to control the establishment of functional and selective sensory motor connections.

Specificity of Neuronal Transcription Factors at Work - The Future

Assembly of the stretch reflex circuit and the establishment of selective monosynaptic connections in the spinal cord appears to be largely hard-wired. The control, modulation and regulation of defined transcriptional programs at successive stages of differentiation and specification of Ia afferents and α-motor neurons is essential to achieve appropriate assembly of the monosynaptic circuit and establishment of selective synaptic connections. The existence of numerous families of regulatory transcription factors, including the ETS family demands for tight regulation of the specificity of expression and action of a specific transcriptional regulator within a defined cellular context such as Ia afferents or α-motor neurons. While molecular properties (such as DNA binding specificity or transcriptional activation properties) can be studied in great detail in heterologous isolated cellular *in vitro* systems, genetic deletion or misexpression of particular transcription factors *in vivo* allows an insight into the biological role of this transcriptional regulator. The identification of true, specific and relevant downstream target genes of a particular member of a family of transcription factors however is not trivial but absolutely essential to develop an understanding of how distinct transcriptional regulators mediate their unique biological response. The target genes of transcription factors represent the next stage in the signaling cascade initiated by a particular transcriptional regulator. Proteins encoded by these target genes are, or regulate, the molecules responsible for a defined cellular response, to transcriptional activity, such as axonal outgrowth, pathfinding and branching, formation of selective synaptic specializations, acquisition of defined neurotransmitter profiles and transmission of neuronal information within neuronal circuits. For these reasons the identification of downstream *in vivo* target genes for individual transcriptional regulators will hopefully greatly contribute to yield exciting information not only about how these transcription factors function mechanistically but also how they regulate and adjust the genetic profile leading to the activation of further distinct molecular signaling pathways within defined biological processes such as the assembly of a functional neuronal circuit.

Experimental Procedures

Molecular Biology

DNA cloning, to construct all plasmids (incl. targeting vectors), and all nucleic acid procedures that were described throughout the experimental studies were carried out using standard cloning protocols (Sambrook et al., 1989).

Generation of Transgenic Mice and Mouse Genetics

Er81^{Pea3} (S. Arber and T.M. Jessell), *Er81*^{Erm}, *Er81*$^{Erm\text{-}Pea3}$ and *Er81*$^{EWS\text{-}Pea3}$ (S. Arber and T.M. Jessell) mice were generated following a similar strategy as described for the generation of *Er81*^{NLZ} mice (Arber et al., 2000). In brief, targeting vectors with cDNAs coding for either *Pea3*, *Erm*, *Erm-Pea3* or *EWS-Pea3* were inserted in frame with the endogenous start ATG into exon 2 of the *Er81* genomic locus and used for homologous recombination in W95 or 129/Ola ES cells. *EWS-Pea3* represents a fusion gene between the amino terminal of EWS and the ETS domain of Pea3 (Urano et al., 1996). The fusion Erm-Pea3 encompasses nucleotides 1-819 of Erm fused in frame to nucleotides 702-1440 of Pea3. Primer pairs to specifically detect *Er81*^{Pea3}, *Er81*^{Erm}, *Er81*$^{Erm\text{-}Pea3}$ and *Er81*$^{EWS\text{-}Pea3}$ alleles were as follows:

Pea3ki (5'): GGGCTGTCGAGGGTAATTAGCTAT (upstream exon 2 in *Er81* locus)
Pea3ki (3'): GACCATCAGCGCTTCGCCCAA (in *Pea3* 5')
Ermki (5'): 5' GAC TCC TCA CTC ACT TCC AGA AC 3' (in *Erm* 5')
Ermki (3'): 5' CTC CTG CTT GAC TTT GCC TTC 3' (in *Erm* 3')
Erm-Pea3ki (5'): 5' GAC TCC TCA CTC ACT TCC AGA AC 3' (in *Erm* 5')
Erm-Pea3ki (3'): 5' C TTC CTG CTT GAT GTC TCC TTC 3' (in *Pea3* 3')
EWS-Pea3ki (5'): CAGCCACTGCACCTACAAGAC (in *EWS* 5')
EWS-Pea3ki (3'): CTTCCTGCTTGATGTCTCCTTC (in *Pea3* 3')

Generation of *Tau*^{mGFP} (M. Sigrist and S. Arber) and *Tau*$^{EWS\text{-}Pea3}$ mice: *lox-STOP-lox-mGFP-IRES-NLS-LacZ-pA* and *lox-STOP-lox-EWS-Pea3-IRES-NLS-LacZ-pA* targeting cassettes were integrated into exon 2 of the *Tau* genomic locus (the endogenous start ATG was removed in the targeting vectors). Membrane-targeted GFP (mGFP) was provided by P. Caroni (see De Paola et al., 2003). ES cell recombinants were screened by Southern blot analysis using the probe in the 5' region as described previously (Tucker et al., 2001). Frequency of recombination in 129/Ola ES cells was ~1:3 for both *Tau* constructs. For the generation of *PV*^{Cre} mice (M. Sigrist and S. Arber), mouse genomic clones were obtained by screening a 129SV/J genomic library (Incyte Genomics). For

details on the genomic structure of the mouse *Parvalbumin* locus see Schwaller et al. (1999). An *IRES-Cre-pA* targeting cassette (Yang et al., 2001) was integrated into the 3' UTR of exon 5 and ES cell recombinants were screened with a 5' probe (oligos: (5') GAGATGACCCAGCCAGGATGCCTC and (3') CTGACCACTCTCGCTCCGGTGTCC; genomic DNA: HindIII digest). The frequency of recombination in 129/OLA ES cells was ~1:20. Recombinant clones were aggregated with blastocysts to generate chimeric founder mice that transmitted the mutant alleles. In all experiments performed in this study animals were of mixed genetic background (129/Ola and C57Bl6). *Thy-1*^{spGFP} transgenic mice were generated (M. Sigrist, P. Caroni and S. Arber) in analogy to De Paola et al., 2003 and for these experiments a strain of mice with early embryonic expression was selected.

Isl1^{Cre} (Srinivas et al., 2001), *Nrg1*^{flox} (Yang et al., 2001), *ErbB2*^{flox} and *HSA-Cre* (Leu et al., 2003), *CRD-Nrg1* (Wolpowitz et al., 2000), *Nrg1*$^{\Delta EGF\text{-}LacZ}$ (Meyer et al., 1997), *TrkC* (Liebl et al., 1997), *Ngn1* (Ma et al., 1999), *Egr3* (Tourtellotte et al., 1998), *Pea3* (Livet et al., 2002), *Bax* (White et al., 1998), *Hb9*^{Cre} (Yang et al., 2001), and *NT-3*^{LacZ} (Farinas et al., 1994) mutant mouse strains have been described. Timed pregnancies were set up to generate embryos of different developmental stages with all genotypes described throughout the study.

Supplemental Note to Isl1^{Cre} *Mutant Mice*

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.

Neuronal Numbers in DRG of Nrg1 Mutant Mice

The absence of Schwann cells from peripheral nerves has been reported to lead to the death of motor and DRG neurons (reviewed by Garratt et al., 2000). Therefore it was evaluated whether the elimination of *Nrg1* from DRG and motor neurons affected the survival of proprioceptive neurons and the profile of gene expression in lumbar DRG. Analysis of the number of PV^+ neurons as well as the level of expression of PV in lumbar DRGs at E16.5 and E18.5 showed no significant difference between $Isl1^{Cre}/Nrg1^{flox/-}$ mutants and wild-type embryos. These findings provide evidence that the remaining Schwann cells in the peripheral nervous system are sufficient to promote the survival of motor and DRG neurons in $Isl1^{Cre}/Nrg1^{flox/-}$ mutant mice. In addition, PV^+ neurons in $Isl1^{Cre}/Nrg1^{flox/-}$ mutants coexpressed the ETS transcription factor ER81, in a manner similar to wild-type mice (Arber et al., 2000), indicating that the absence of *Nrg1* from DRG neurons does not influence expression of PV or ER81 in proprioceptive afferents.

Transcriptional Transactivation Assays
(according to T. Portmann, Diploma Thesis 2003)

The following plasmids were used for transcriptional transactivation assays: pRc/RSV (Invitrogen), pRc/RSV-Pea3, pRc/RSV-Erm, pRc/RSV-Er81, pRc/RSV-Erm-Pea3, pRc/RSV-EWS-Pea3, pTP-5xETS, pTP-5xETSmut, pTP-5xETS/Runx. pRc-RSV-Pea3, pRc-RSV-Erm, pRc-RSV-Er81, pRc-RSV-Erm-Pea3 and pRc/RSV-EWS-Pea3 were obtained by insertion of the cDNAs for *Pea3*, *Erm*, *Er81*, *Erm-Pea3*, or *EWS-Pea3* (gift from J. A. Hassell) into pRc/RSV. pTP-5xETS was constructed by inserting a cassette of five repetitive copies of high affinity Pea3 binding sites (5'-GCCGGAAGC -3'; Mo et al., 1998; Bojovic and Hassell, 2001) into a modified version of pTK-Luc. pTP-5xETSmut was generated as pTP-5xETS but using a mutated complement of the Pea3 binding sites (5'- GCCTATGGC -3'). pTP-5xETS/Runx was generated as pTP-5xETS but with additional integration of two optimized Runx binding sites (for details and further information see T. Portmann, Diploma Thesis, 2003). A control plasmid to normalize for transfection efficiency (placZ) and pTK-Luc were a gift from D. Kressler. COS-7 cells were co-transfected with 1-1.2µg of total DNA including one of the effector plasmids pRc/RSV-empty, pRc-RSV-Pea3, or pRc/RSV-EWS-Pea3, respectively; one of the reporter plasmids pTP-5xETS or pTP-5xETSmut and placZ. Cells were harvested after 25h and processed for assays to determine luciferase and LacZ activity as described previously (Kressler et al., 2002). Luciferase values normalized to LacZ activity are referred to as luciferase units. Data shown represent the mean ± SEM of at least 7 values from 3 independent experiments performed.

In Situ Hybridization and Immunohistochemistry

For *in situ* hybridization analysis, cryostat sections were hybridized using digoxigenin-labeled probes (Schaeren-Wiemers and Gerfin-Moser, 1993) directed against mouse *Egr3*; *Pea3* (Livet et al, 2002); *Gfrα1* and *Ret* (Haase et al., 2002); *ChAT* and *RALDH2* (Arber et al., 1999); *Erm* (cDNA kindly provided by JA. Hassell); *Isl1* (Arber et al., 2000); *Ig-Nrg1* and *CRD-Nrg1* specific Nrg1 isoforms (Wolpowitz et al., 2000); *TrkA*, *TrkB*, and rat *TrkC*, respectively (gift from L.F. Parada). For generation of an *Egr3* specific probe, a fragment encompassing nucleotides (451-1071) of the *Egr3* coding sequence (Genebank accession number AF132128) was amplified from genomic DNA by PCR. Digoxigenin-labeled probes directed against candidate genes, found as a result of Affymetrix gene chip experiments (see below), were generated using plasmid preparations of clones from I.M.A.G.E.: *Aquaporin* (Genebank accession number BC007125), *Dok4* (Genebank accession number BC004705), *Rgs4* (Genebank accession number BC003882), *CD44* (Genebank accession number BC005676), *Basonuclein* (Genebank accession number BQ958831), *sFRP2* (Genebank accession number BC014722), *sFRP3* (Genebank accession number BC016884), *Pbx3b* (Genebank accession number BE572809), *Tachykinin* (Genebank accession number AA689911).

Antibodies used in this study were: rabbit anti-Egr3, rabbit anti-Gap43, rabbit anti-S100, rabbit anti-Er81 (c-term), rabbit anti-Pea3 (c-term), rabbit anti-PV (Arber et al., 1999; Arber et al., 2000); rabbit anti-ErbB3 (Santa Cruz, SC285); rabbit anti-ErbB4 (#618; Zhu et al., 1995); rabbit anti-eGFP (Molecular Probes); rabbit anti-Calbindin, rabbit anti-Calretinin (SWANT); rabbit anti-Substance P (Peninsula Laboratories Inc.); rabbit anti-CGRP (Chemicon Int.); rabbit anti-Rhodamine (Molecular Probes); rabbit anti-Runx1 and rabbit anti-Runx3 (gift from M. Sigrist, I. Kramer and S. Arber); rabbit anti-Hb9 (Arber et al., 1999); rabbit anti-TrkA (Huang et al., 1999; Arber et al., 2000); rabbit anti-p75 (Weskamp et al., 1991); mouse anti-neurofilament (3A10); sheep anti-eGFP (Biogenesis Ltd); goat anti-PV (SWANT); goat anti-TrkC (Huang et al., 1999; Arber et al., 2000); goat anti-LacZ, guinea pig anti-Isl1 (Arber et al, 2000); chick anti-TrkB (Huang et al., 1999).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) to detect apoptotic cells in E13.5 DRG on cryostat sections was performed as described by the manufacturer (Roche). Quantitative analysis of $TUNEL^{+}$ DRG cells was performed essentially as described (White et al., 1998).

For visualization of enzymatic β-Galactosidase activity in whole mount, stainings of the entire spinal cord or *gluteus maximus* muscle were performed as described (Arber et al., 1999; Arber et al., 2000).

For anterograde tracing experiments to visualize projections of sensory neurons, rhodamine-conjugated dextran (Molecular Probes) was injected into single lumbar (L3) DRG at E13.5 or applied to whole lumbar dorsal roots (L3) at P5 using glass capillaries (according to E. Vrieseling). After injection, preparations were incubated for 2-3h (E13.5) or over night (P5).

Cryostat sections were processed for immunohistochemistry as described (Arber et al., 2000) using fluorophore-conjugated secondary antibodies (1:1000, Molecular Probes). 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.

Quantification of PV^+ and $Runx3^+$ Cells in Lumbar DRG

For quantification of PV^+ and $Runx3^+$ cells in lumbar DRG, every third or sixth section (12μm) was evaluated. Sections, blind of genotype, were subjected to confocal microscopy and the number of either PV^+ or $Runx3^+$ cells was determined for every section. The average number of PV^+ or $Runx3^+$ cells/section was determined for every L1 to L6 DRG individually and the resulting 'average counting indices' served as primary data units for statistical analysis. The unequal size of DRG as a result of sectioning from rostral to caudal segmental levels was taken into account in the distribution of the 'average counting indices' on charts (left is rostral, right is caudal in the arrays of data points) shown in chapter eight.

Gene Chip Experiments

For gene chip experiments total mRNA was extracted and purified from single DRG collected from embryos at E16.5 using TRIzol Reagent (GibcoBRL, Life Technologies) and RNeasy kit (Qiagen). Analysis was performed with U74Av2/U74Bv2 GeneChips (Affymetrix). Changes in gene expression were assessed using Affymetrix Microarray Suite v5 and GeneSpring 4.2.1 (Silicon Genetics). Absolute values described in this study represent results obtained from three individual replicates/genotype.

Western Blot Analysis

Lumbar DRG from E16.5 embryos were isolated, mechanically disrupted, homogenized using glass beads (Sigma) and lysed in standard lysis buffer supplemented with protease and phosphatase inhibitors as described (Markus et al., 2002b). Protein extracts were resolved by SDS-PAGE, and immunoblotting was performed using antibodies against Akt, phospho-Akt, CREB, phospho-CREB, Bax, Bcl-xl (Cell Signaling Technology), and Bcl-2 (BD PharMingen). For quantification, films (X-OMAT AR, Kodak) were scanned and densitometry was performed using IMAGE QUANT 5.2.

In Vitro Cultures of Dorsal Root Ganglia

Individual lumbar DRG were dissected from E13.5 embryos and placed on Matrigel (BD Biosciences) coated coverslips in DMEM/F12 (Gibco), 2mM L-Gln (Gibco), N2 (Gibco), 1mg/ml BSA (Sigma) without neurotrophins or supplemented with either NGF (100ng/ml, Gibco) or NT-3 (20ng/ml, Sigma). DRG explants ($n \geq 20$ for each condition) were cultured for 48h, processed for immunocytochemistry and analyzed using confocal microscopy.

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Appendix

Ich erkläre, dass ich die Dissertation, ***Molecular Mechanisms of Neuronal Circuit Assembly in the Vertebrate Spinal Cord***, nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingericht habe.

Curriculum Vitae

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Education and Research Experience

Since 04/2000	PhD studies at Biozentrum, Dept. Cell Biology, University of Basel and Friedrich Miescher Institute, Basel on ***Molecular Mechanisms of Neuronal Circuit Assembly in the Vertebrate Spinal Cord*** (Supervision: Prof. Dr. S. Arber).
04/2000	Diploma in Bio II, dipl. phil. II (Biochemistry).
10/1998 - 04/2000	Diploma thesis at Biozentrum, Dept. Biochemistry, University of Basel on ***Functional Analysis of Arc35/End9p, the 35 kDa Subunit of the Arp2/3 Complex in Saccaromyces cerevisiae*** (Supervision: Prof. Dr. H. Riezman).
10/1995 - 04/2000	Diploma studies in Bio II (Molecular Biology) at Biozentrum, University of Basel, Switzerland.
08/1991 - 12/1994	Matura Typus C, Gymnasium Muttenz, Switzerland.

Extracurricular Activities

03/2002 - 02/2004	Chair of VSB
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Teaching

Since 09/2003	Supervision of diploma student (C. Laengle)
06/2003	Lab assistance (Neurobiology) for Bio II students
10/2002 - 07/2003	Supervision of diploma student (T. Portmann)
10/2000 - 02/2002	Tutorial for Bio II students (Introduction in Biology)
02/1999	Lab assistance (Biochemistry) for Bio II students

Scientific Presentations, Meetings and International Congresses

Poster Presentations:
- Society for Neuroscience 33rd Annual Meeting, 2003, New Orleans, USA
- FMI Conference: Formation and Function of Neuronal Circuits, 2003, Ascona, Switzerland
- EMBO Workshop: The Assembly of Neural Circuits, 2003, Varenna, Italy
- Gordon Research Conference on Neurotrophic Factors, 2003, Newport, USA
- Society for Neuroscience 32nd Annual Meeting, 2002, Orlando, USA
- ELSO Meeting, 2000, Geneva, Switzerland

Talks:
- Swiss-Japanese Meeting, Developmental Biology, 2003, Ohito, Japan
- FMI Annual Meeting, 2003, Pontresina, Switzerland
- FMI-Novartis Neuroscience Joint Meeting, 2003 Basel, Switzerland
- FMI Annual Meeting, 2002, Luzern, Switzerland

Invited Seminar:
- Colloqium with B. Winsor, 2000, IBMC, Strasbourg, France

Publications

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Scientific Communications

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