# Molecular Pathways of Proprioceptive Dorsal Root Ganglion (DRG) Sensory Neuron Specification

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| ACh | acetylcholine |
| Ala | alanine |
| AML | acute myeloid leukemia |
| ARTN | artemin |
| Bax | Bcl2-associated X protein |
| Bcl2 | B-cell leukemia/ lymphoma 2 |
| BDNF | brain derived neurotrophic factor |
| BMP | bone morphogenetic protein |
| Car2 | carbonic anhydrase 2 |
| Cbf | core binding factor |
| CGRP | calcitonin gene-related peptide |
| CNS | central nervous system |
| Cre | causes recombination |
| CREB | cAMP response element binding protein |
| Cys | cysteine |
| DiI | 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate |
| DMEM | Dulbecco's modified Eagle medium |
| DRG | dorsal root ganglion/ganglia |
| Egr3 | early growth response 3 |
| Er81/Etv1 | ets related protein 81/ ets variant gene 1 |
| Erm/Etv5 | ets related molecule Pea3-like/ ets variant gene 5 |
| EST | expressed sequence tag |
| ETS | E twenty-six/ erythroblastoma transformation specific |
| GDNF | glial cell line derived neurotrophic factor |
| EGFP | enhanced green fluorescent protein |
| GFRα | GDNF family receptor α |
| Gbb | Glass bottom boat |
| Glu | glutamate |
| GPCR | G protein-coupled receptor |
| GPI anchor | glycosyl-phosphatidyl-inositol anchor |
| GTO | Golgi tendon organ |
| IB4 | isolectin B4 |
| Ig | immunoglobulin |
| IRES | internal ribosome entry side |
| Isl1 | islet 1 |
| LCM | lateral motor column |
| *lox*P | locus of crossing over in P1 |
| Mad | Mothers against dpp |
| MAPK | mitogen activated protein kinase |

| | |
|---|---|
| MARCKS | myristoylated alanine rich protein kinase C substrate |
| Mash1 | mouse achaete-scute homologue 1 |
| Me6/Gpr64 | mouse epididymis-specific protein 6/ G protein-coupled receptor 64 |
| MMC | median motor column |
| Mrg | Mas-related gene |
| Myc | myelocytomatosis oncogene |
| NGF | nerve growth factor |
| Ngn | neurogenin |
| NLS | nuclear localization signal |
| NHO | neurohemal organs |
| NMJ | neuromuscular junction |
| Npy | neuropeptide Y |
| Nrg1 | neuregulin 1 |
| NT | neurotrophin |
| Opn/Spp1 | osteopontin/ secreted phosphoprotein 1 |
| PCR | polymerase chain reaction |
| PEBP2 | polyoma enhancer-binding protein 2 |
| Pea3/Etv4 | polyomavirus enhancer activator 3/ ets variant gene 4 |
| PFA | paraformaldehyde |
| Phox2a/b | paired-like homeobox 2a/b |
| PI3K | phosphatidylinositol-3-kinase |
| PNS | peripheral nervous system |
| Pthr1 | parathyroid hormone receptor 1 |
| PTHrP | parathyroid hormone-related peptide |
| PV | parvalbumin |
| RD | runt domain |
| Runx | runt related transcription factor |
| SCG | superior cervical ganglion |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| Ser | serine |
| SNSR | sensory neuron-specific G protein-coupled receptor |
| Syp | synaptophysin |
| Tac1 | tachykinin 1 |
| TGFβ | transforming growth factor β |
| Thr | threonine |
| Thy1 | thymus cell antigen1, theta |
| TM | transmembrane |
| TLE | transducin-like enhancer of split |
| Trk/Ntrk | tyrosine kinase, receptor/ neurotrophic tyrosine kinase, receptor |
| UTR | untranslated region |
| Wit | Wishful thinking |
| Wnt | wingless-related MMTV integration site |

# ZUSAMMENFASSUNG

Sensorische Neurone der Dorsalwurzelganglien (DRG) können aufgrund der Expression spezifischer Rezeptor-Tyrosinkinasen, die als Rezeptoren neurotropher Faktoren bekannt sind, in verschiedene neuronale Subpopulationen klassifiziert werden. Noziozeptive und thermozeptive DRG Neurone exprimieren den Rezeptor für Nervenwachstumsfaktor (nerve growth factor, NGF), die Rezeptor-Tyrosinkinase TrkA, mechanorezeptive DRG Neurone exprimieren TrkB, den Rezeptor für brain derived neurotrophic factor (BDNF) und Neurotrophin 4/5 (NT4/5), während propriozeptive DRG Neurone den Neurotrophin 3 (NT3)-Rezeptor TrkC exprimieren. Des weiteren exprimiert eine Subpopulation von DRG Neuronen die Rezeptor-Tyrosinkinase Ret, die Signale der glial cell line derived neurotrophic factor (GDNF)-Proteinfamilie überträgt.

Die Expression der Rezeptoren neurotropher Faktoren spielt eine wichtige Rolle, nicht nur im Vermitteln des Überlebens von Subpopulationen sensorischer DRG Neurone, sondern auch in der Kontrolle der Ausbildung phänotypischer Eigenschaften. Die Expression eines bestimmten neurotrophen Faktor-Rezeptors ist mit der Ausbildung von einzigartigen funktionellen Eigenschaften und neuronalen Verknüpfungen während der Embryonalentwicklung verbunden. Durch gleichzeitige Eliminierung des *Bax* (*Bcl2-associated X protein*)-Gens, eines Mitglieds der pro-apoptotischen Bcl2 (B-cell leukemia/ lymphoma 2)-Genfamilie, und des NT3 kodierenden Gens konnten wir zeigen, dass NT3 aus dem Muskel für die Induktion der Expression des ETS Transkriptionsfaktors Er81 in TrkC-positiven propriozeptiven DRG Neuronen verantwortlich ist. In Abwesenheit des NT3-Signals ist die Expression von Er81 in propriozeptiven DRG Neuronen stark reduziert, weshalb propriozeptive Afferenzen nicht ins Ventralhorn des Rückenmarks projizieren und daher keine monosynaptischen Verbindungen mit spinalen α-Motorneuronen ausbilden. Folglich wird das periphere NT3-Signal für die Bildung von sensorisch-motorischen Verknüpfungen während der Entwicklung durch Induktion von Er81 in propriozeptiven Neuronen benötigt.

Im Gegensatz zu den nachgeschalteten molekularen Signalkaskaden, die durch neurotrophe Faktoren ausgelöst werden, ist relativ wenig über die molekularen

Mechanismen bekannt, die der selektiven Expression der Rezeptoren neurotropher Faktoren in differenzierenden DRG Neuronen zugrunde liegen. Bei der mittels eines differentiellen Screens durchgeführten Suche nach neuen in differenzierenden DRG Neuronen exprimierten Markergenen identifizierten wir den Runt-verwandten Transkriptionsfaktor Runx3. In frühen Entwicklungsstadien ist die Runx3 Expression eng mit einer Subpopulation sensorischer DRG Neurone assoziiert, die alleinig TrkC exprimiert, nicht aber TrkA, TrkB und Ret. Durch Anwendung genetischer Manipulationen in der Maus haben wir herausgefunden, dass Runx3 spezifisch in TrkC exprimierenden DRG Neuronen für die Förderung eines neuronalen Phänotyps assoziiert mit propriozeptiver Identität durch die Suppression der TrkB-Expression in angehenden propriozeptiven DRG Neuronen benötigt wird, wodurch Runx3 entscheidend an der Konsolidierung eines propriozeptiven Entwicklungsschicksals durch die gezielte Auslöschung eines alternativen neuronalen Entwicklungspotentials beteiligt ist. Im Gegensatz hierzu ist Runx3 nicht ausreichend, um TrkA exprimierende kutane DRG Neurone in propriozeptive sensorische Neurone zu konvertieren. Zusammen zeigen diese Ergebnisse, dass der Status der Runx3-Expression durch Kontrolle der Expression von Rezeptoren neurotropher Faktoren eine Schlüsselrolle in der korrekten Differenzierung propriozeptiver Afferenzen zukommt.

# SUMMARY

Dorsal root ganglia (DRG) sensory neurons can be classified into distinct neuronal subpopulations based on the expression of specific receptor tyrosine kinases known as neurotrophic factor receptors. Nociceptive and thermoceptive DRG neurons express the nerve growth factor (NGF) receptor tyrosine kinase TrkA, mechanoreceptive DRG neurons express TrkB, the receptor for brain derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT4/5), whereas proprioceptive DRG neurons express the neurotrophin 3 (NT3) receptor TrkC. Moreover, a subpopulation of DRG neurons expresses the receptor tyrosine kinase Ret mediating signaling in response to glial cell line derived neurotrophic factor (GDNF) family proteins.

Neurotrophic factor receptor expression is known to play important roles not only in mediating survival of DRG sensory neuron subpopulations, but also in controlling the acquisition of phenotypic traits. The choice to express a particular neurotrophic factor receptor is associated with the establishment of unique functional properties and patterns of connectivity during embryonic development. By eliminating the pro-apoptotic Bcl2 (B-cell leukemia/ lymphoma 2) family member gene *Bax* (*Bcl2-associated X protein*) concomitant with the gene encoding NT3 we could show that muscle derived NT3 is responsible for inducing expression of the ETS transcription factor Er81 in TrkC positive proprioceptive DRG neurons. In absence of NT3 signaling, Er81 expression is severely reduced in proprioceptive sensory neurons, and therefore proprioceptive afferents fail to project into the ventral horn of the spinal cord and to establish monosynaptic connections with spinal α-motor neurons. Hence, peripheral NT3 signaling is required for establishment of sensory-motor connectivity during development via induction of Er81 expression in proprioceptive DRG neurons.

In contrast to the downstream molecular signaling events elicited by neurotrophic factors, very little is known about the molecular mechanisms underlying the selectivity of neurotrophic factor receptor choice in early differentiating DRG sensory neurons. In a differential screen aimed at the identification of novel marker genes expressed by differentiating DRG neurons we identified the runt related transcription factor Runx3. At

early developmental stages, Runx3 expression is tightly associated with a sensory neuron subpopulation that expresses TrkC alone, but excluded from TrkA, TrkB and Ret expressing DRG sensory neurons. Using genetic manipulations in the mouse, we found that Runx3 is specifically required within TrkC expressing DRG neurons to promote a neuronal phenotype associated with a proprioceptive identity by suppressing TrkB expression within presumptive proprioceptive DRG neurons, thus contributing to the consolidation of a proprioceptive fate through the coordinate extinction of an alternative neuronal fate. In contrast, Runx3 is not sufficient to promote conversion of TrkA expressing cutaneous DRG neurons into proprioceptive sensory neurons. Together, these findings demonstrate that the status of Runx3 expression is of key importance for the proper differentiation of proprioceptive afferents by controlling neurotrophic factor receptor choice in DRG neurons.

# CHAPTER 1

## INTRODUCTION

## THE MONOSYNAPTIC STRETCH REFLEX CIRCUIT:
### A MODEL SYSTEM FOR STUDYING NEURONAL SPECIFICATION AND CIRCUIT FORMATION

# INTRODUCTION

Understanding the functional complexity of the mature mammalian central nervous system (CNS) is one of the most challenging tasks within the broad field of traditional and modern neuroscience. The tremendous complexity of the higher mammalian brain has, however, so far prevented a comprehensive detailed functional and molecular analysis of this organ.

A first step towards a deeper understanding of mature brain function is to understand the basic neuronal wiring principles, which underlie and govern functional properties of the adult nervous system and already start to be established during embryonic development. Identifying the molecular determinants that control neuronal subtype specification and subsequent neuronal circuit formation will thus be of great importance in order to be able to decipher and understand the functioning of the mature nervous system. However, acquiring a detailed understanding about neuronal subpopulation specific characteristics such as defined temporal gene and protein expression profiles will not suffice, as the real challenge consists in understanding the functional interrelation and specific developmental outputs generated by the specific molecular determinants.

To functionally address the complex nature of neuronal subpopulation specification and circuit formation it has often proven useful to study neuronal development not directly in the highly complex mammalian central nervous system, but instead to focus on comparatively simple model systems, which can be found for example in the peripheral nervous systems (PNS) of mammals and lower vertebrates as well as in arthropods or worms (Chalfie et al., 1985; Komiyama et al., 2003; Tayler and Garrity, 2003; Tsalik and Hobert, 2003; Stowers, 2004; Gray et al., 2005; Stockinger et al., 2005).

An ideally suited model system for studying neuronal circuit establishment in the mammalian nervous system is the developing monosynaptic stretch reflex circuit connecting proprioceptive Ia sensory neurons of the peripheral nervous system with spinal α-motor neurons in the central nervous system (Chen et al., 2003). The advantages of studying the formation of this fairly simplistic neuronal circuit are multiple. First, it has been the focus of many studies in the past, both anatomical as well as electrophysiological, and thus its' basic neuronal components (proprioceptive sensory

neurons and spinal α-motor neurons) have been extensively described and reviewed previously (Eccles et al., 1957; Brown 1981; Scott, 1992; Clarac et al., 2000; Chen et al., 2003). Secondly, it is well suited for experimental manipulations, since it is easily accessible from the periphery making it an ideal object for anatomical tracing experiments and studies addressing as different questions as regulation of axon guidance decisions or control of specificity of synapse formation, elimination and maintenance.

## THE MONOSYNAPTIC STRETCH REFLEX CIRCUIT - A MODEL SYSTEM

Proprioception is defined as the sense of the orientation of the limbs and other body parts in space with respect to each other allowing complex and yet coordinated movements to be carried out. As part of the somatosensory system, it relies on the correct processing of internal and external sensory inputs obtained from sensory neurons relaying information from the body's periphery using specialized sensory organs and receptors present in the skin, inner ear, skeletal muscles, tendons and joints.

One classic subgroup of proprioceptors resides within the peripheral dorsal root ganglia (DRG), which lie on both sides immediately adjacent to the spinal cord and are present in each segmental level along the entire rostro-caudal axis. Proprioceptive DRG sensory neurons are characterized by a large cell body diameter (60-120 μm) and expression of the neurotrophin receptor TrkC/Ntrk3. They comprise three major subclasses categorized as Ia, Ib and II proprioceptive neurons differing not only with respect to their specific function but also displaying differences in their peripheral and central connectivity. However, detailed knowledge about how these different proprioceptive subclasses are specified during embryonic development is missing, as there are no molecular markers available that would allow distinguishing individual proprioceptive subgroups.

Proprioceptive DRG neurons are pseudo-unipolar primary sensory neurons with a peripheral axon branch projecting towards selective skeletal limb muscles where they terminate to innervate specific types of mechanoreceptors (Figure 1). Group Ia and II proprioceptive neurons innervate muscle stretch sensitive sensory organs termed muscles spindles that lie in parallel to extrafusal skeletal muscle fibers innervated by α-motor

neurons. In contrast, Ib proprioceptive neurons innervate stretch receptors called Golgi tendon organs (GTOs) lying in series to skeletal muscle fibers at the myo-tendinous junctions of mammalian skeletal limb muscles (Zelena, 1994).

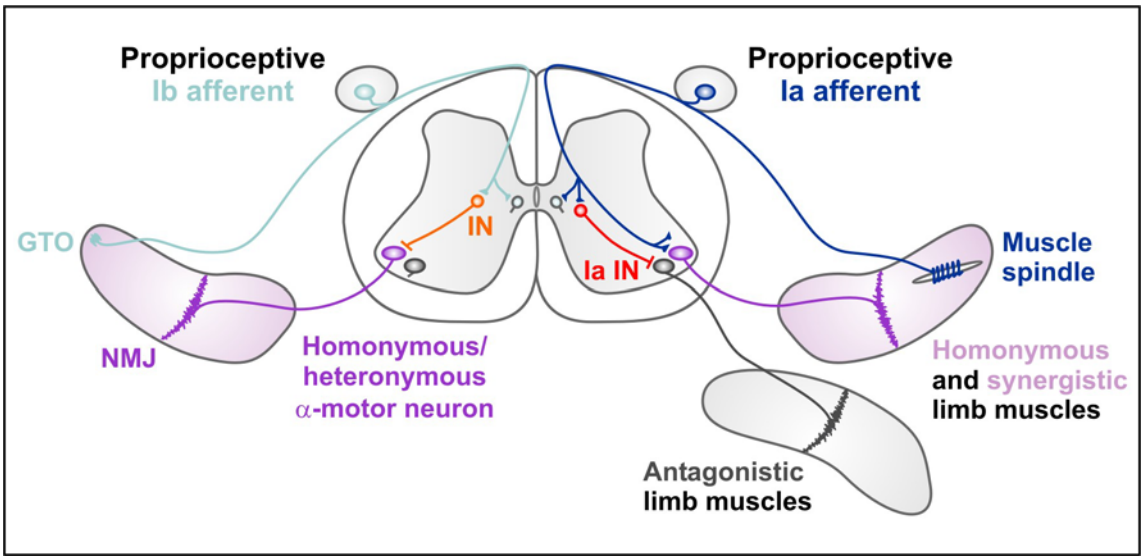


**Figure 1.** Spinal reflex circuitry of proprioceptive sensory neurons

Central and peripheral projections of group Ia (right: blue) and Ib (left: light blue) proprioceptive DRG neurons. **Left:** group Ib proprioceptive afferents peripherally innervate Golgi tendon organs (GTOs) at the myo-tendinous junctions of skeletal limb muscles and centrally project to the intermediate spinal cord to form synapses with interneurons (IN: orange), thereby indirectly connecting with spinal α-motor neurons (purple) in the ventral horn of the spinal cord.
**Right:** group Ia afferents peripherally innervate muscle spindles and centrally form direct monosynaptic connections to homo- and heteronymous α-motor neurons (purple). α-motor neurons projecting to antagonistic muscles (grey) are innervated in a di-synaptic fashion via Ia inhibitory interneurons (Ia IN: red). Abbreviations: IN = interneuron; NMJ = neuromuscular junction. (Adapted from Arber et al., 2000.)

Centrally, proprioceptive afferents project through the dorsal root towards the spinal cord, which they reach by about E10.5, then bifurcate and extend rostro-caudally in the dorsal part of the dorsal funiculus on the dorsal side of the spinal cord before sending out interstitial axon collaterales by about E12.5 at regular intervals (Ozaki and Snider, 1997). These axon collaterals then project from the dorsal part of the spinal cord towards the intermediate region of the spinal cord, where they ramify to from synapses with corresponding target neurons (Figure 1). In addition, Ia proprioceptive neurons have the capacity to project down to the ventral horn of the spinal cord, where they establish direct monosynaptic connections with spinal α-motor neurons projecting to the same muscle in

the periphery (homonymous α-motor neurons) or to functionally related, synergistic muscle groups (heteronymous α-motor neurons). In contrast, Ia proprioceptive afferents usually do not form any monosynaptic connections with α-motor neurons innervating muscles of antagonistic function. Instead, these are innervated in a di-synaptic manner via inhibitory Ia interneurons located in the intermediate region of the spinal cord, which receive direct monosynaptic input from Ia afferent collaterals (Figure 1).

In contrast to proprioceptive neurons, whose cell bodies never cluster together within the DRG but rather are intermingled with sensory neurons of different functional modalities, functionally linked α-motor neurons projecting to the same muscle in the periphery have their cell bodies grouped within the ventral horn of the spinal cord in so-called motor neuron pools. Motor neuron pools are located within longitudinal motor columns that extend in parallel to the rostro-caudal axis of the spinal cord. Motor neurons innervating skeletal muscle targets in the limbs are present in the lateral motor column (LCM), which exists only at the fore- and hindlimb levels of the spinal cord. In contrast, motor neurons innervating axial and body wall muscle targets are found in the median motor column extending along the entire axis of the spinal cord (MMC; Jessell, 2000).

The peripheral synapses formed between α-motor neurons and extrafusal muscle fibers are cholinergic synapses called neuromuscular junctions (NMJ) that are among the most intensively studied types of synapses of the entire nervous system. When a motor axon reaches its target muscle it ramifies to form synapses with multiple individual muscle fibers, so that in the mature state each extrafusal muscle fiber receives input from only a single α-motor neuron, which is accomplished by a process of synaptic competition and synapse elimination taking place during normal postnatal development (Sanes and Lichtman, 2001). The entire ensemble of all muscle fibers innervated by a single motor axon together with the axon is called a motor unit.

When a skeletal limb muscle is stretched this does not only lead to a direct lengthening of the extrafusal muscle fibers, but it will also stretch intrafusal muscle fibers inside the muscle spindles, thereby activating Ia proprioceptive afferents. As a consequence, the information from the periphery of the muscle stretch is relayed to the central nervous system in form of action potentials traveling along the Ia afferent axonal collaterals all the way towards the ventral horn of the spinal cord. Subsequent release of L-glutamate

(L-Glu) at the Ia-α-motor neuron synapses then causes direct monosynaptic activation of the corresponding α-motor neurons, which if sufficiently strong activated, fire action potentials inducing contraction of the skeletal muscle in the periphery via release of acetylcholine (ACh), hence bringing the muscle back to its initial state.

## EMBRYONIC DRG SENSORY NEURON SUBPOPULATIONS

Proprioceptive neurons comprise only about 10-20% of all DRG neurons depending on the respective segmental level (Table 1). The majority of DRG sensory neurons are thermoceptive and nociceptive neurons conveying noxious (painful) stimuli from the periphery to the central nervous system. Neurons belonging to this subclass of peripheral sensory neurons comprise 70-80% of all DRG neurons and are characterized by a small cell body diameter and peripheral axon projections innervating specialized sensory receptors in the skin (Table 1). In contrast to proprioceptive DRG neurons, nociceptive and thermoceptive DRG neurons centrally innervate target neurons within the dorsal horn of the spinal cord.

All classes of DRG sensory neurons arise from neural crest stem cells in response to Wnt/β-Catenin signaling (Lee et al., 2004). Migratory neural crest cells emanating from the dorsal neural tube during early embryonic development settle adjacent to the neural tube to condense and form the developing DRG. Subsequent sensory neurogenesis has been shown to occur in two overlapping waves of neurogenesis (Ma et al., 1999), which depend on the combinatorial activities of two proneural genes encoding the basic-helix-loop-helix transcription factors neurogenin 2 (Ngn2) and neurogenin 1 (Ngn1) controlling the generation of distinct subpopulations of DRG sensory neurons (Bertrand et al., 2002). While proprioceptive and mechanoreceptive sensory neurons are generated from ngn2-dependent progenitors, nociceptive and thermoceptive sensory neurons are generated slightly later from ngn1-derived progenitors.

Different classes of DRG sensory neurons do not only display unique central and peripheral connectivity but they also can be distinguished by the selective expression of different neurotrophic factor receptors (Farinas, 1999), which comprise a special class of

high-affinity receptor tyrosine kinases (Trks). At least three major subgroups of embryonic DRG sensory neurons can be defined by virtue of distinct neurotrophic factor receptor phenotypes (Table 1).

**Table 1:** Major subclasses of embryonic DRG sensory neurons and their characteristics

| Nociceptive and thermoceptive neurons | Mechanoreceptive neurons | Proprioceptive neurons |
|---|---|---|
| TrkA positive | TrkB positive | TrkC positive |
| NGF dependent | BDNF/NT4/5 dependent | NT3 dependent |
| small diameter | large-intermediate diameter | large diameter |
| 70-80%* | 6-10%* | 10-20%* |

* Relative percentage of all DRG sensory neurons varies with respective segmental level

These consist in nociceptive and thermoceptive neurons expressing the nerve growth factor (NGF) receptor TrkA, mechanoreceptive neurons expressing TrkB, the receptor for brain derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT4/5), and finally proprioceptive neurons expressing the neurotrophin 3 (NT3) receptor TrkC (Bibel and Barde, 2000; Huang and Reichardt, 2001, 2003). Moreover, DRG neurons expressing the receptor tyrosine kinase Ret, which functions as an essential co-receptor for signaling in response to glial cell line derived neurotrophic factor (GDNF) family proteins, have been identified but their exact sensory modality and axonal projection patterns still remain to be determined in detail (Molliver et al., 1997).

Neurotrophic factor receptor signaling is essential for mediating neuronal survival of selective neuronal subpopulations during development by preventing the induction of programmed cell death through activation of anti-apoptotic signaling pathways such as the phosphatidylinositol-3-kinase (PI3K) and Akt/PKB signaling pathway (Bibel and Barde, 2000; Huang and Reichardt, 2001, 2003). Interestingly, work from the past few years has revealed additional important functions for neurotrophic factor receptor signaling, both during neuronal differentiation where it was shown to play instructive roles in neuronal specification as well as in the mature nervous system where it is

involved in the expression of synaptic plasticity (McAllister et al., 1999; Schuman, 1999; Huang and Reichardt, 2001, Mendell et al., 2001).

## ROLE OF ETS TRANSCRIPTION FACTORS IN NEURONAL CIRCUIT ASSEMBLY

While fairly little is known about the molecular mechanisms controlling expression of distinct neurotrophic factor receptors in different subpopulations of DRG neurons, some of the molecular mechanisms contributing to the establishment of the monosynaptic stretch reflex circuit have been revealed in the past (Lin et al., 1998; Arber et a., 2000; Hippenmeyer et al., 2002; Inoue et al., 2002; Levanon et al., 2002; Chen et al., 2003). Generally, neuronal circuit assembly can be divided into three major developmental steps. First, the most basic elements of any given neuronal circuit, the different neuronal cell types participating in it, need to be specified correctly. This is thought to rely to a great extend on cell-intrinsic determinants already established at the neuronal precursor stage controlling important steps of early neuronal specification such as neuronal migration and initial neurite outgrowth towards the target regions (Sommer et al., 1995; Edlund and Jessell, 1999; Jessell, 2000; Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002). Second, once neurons have started to send out axons to navigate to their final target areas, which can be located at far distances from the cell body position, they encounter a multitude of different external cues that need to be functionally integrated, both spatially and temporally, thereby influencing further neuronal differentiation steps. Once the target region has been reached axonal branching occurs to allow correct innervation of specific target layers with a given target. The last major step towards formation of functional neuronal circuits consists in the process of specific target cell recognition and initiation of synapse formation, a process that is likely to involve homophilic interactions between cell surface molecules expressed by the pre- and postsynaptic cell (Price et al., 2002; Salinas and Price, 2005). In summary, acquisition of a distinct neuronal fate and specificity in neuronal circuit formation seem to occur via progressive activation of defined genetic programs during development inducing further differentiation steps and consolidation of neuronal identity.

The ETS (E twenty-six/ erythroblastoma transformation specific)-domain transcription factor ets related protein 81 (Er81) has been shown to control a late step in the formation of the monosynaptic stretch reflex circuit (Arber et al., 2000). In *Er81*$^{-/-}$ mice, Ia proprioceptive neurons fail to establish normal axonal projections into the ventral horn of the spinal cord and instead terminate prematurely in the intermediate spinal cord (Figure 2). As a consequence, the great majority of functional monosynaptic connections with spinal α-motor neurons is not formed during development and homozygous mutants are characterized by a strong ataxic phenotype (Figure 2).

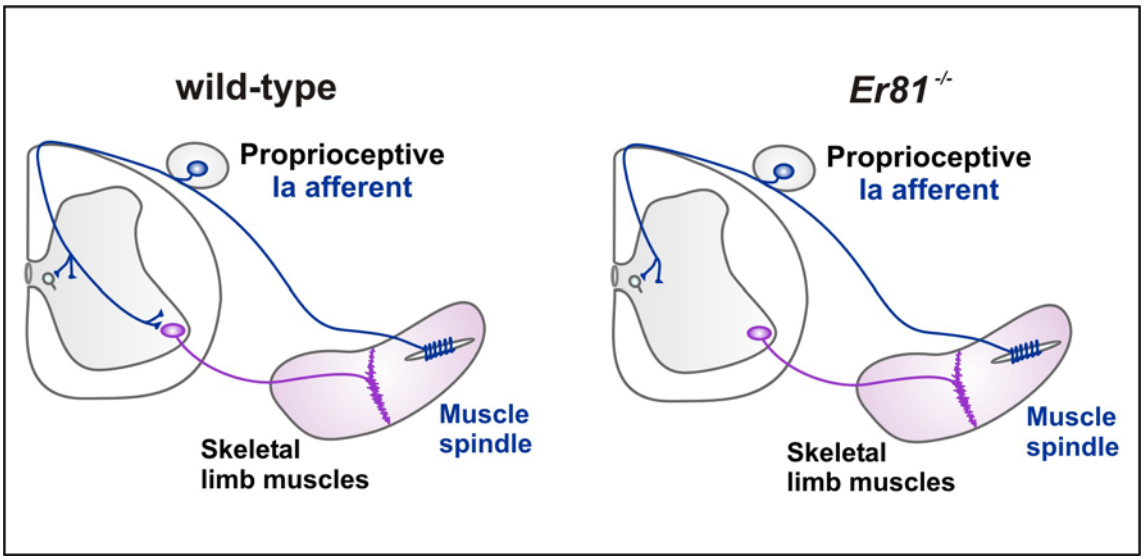


**Figure 2.** Central connectivity defects in *Er81* mutant mice

Peripheral projections of group Ia (dark blue) proprioceptive DRG neurons are normal in *Er81*$^{-/-}$ mice, but central projections to the ventral horn of the spinal cord fail to be established. **Left:** wild-type spinal reflex circuitry of group Ia proprioceptive sensory neurons, which innervate muscle spindles in the periphery and centrally form direct monosynaptic connections to homo- and heteronymous α-motor neurons (purple) in the ventral horn of the spinal cord. **Right:** in *Er81*$^{-/-}$ mice group Ia proprioceptive sensory neurons normally innervate muscle spindles in the periphery, but centrally fail to project into the ventral horn of the spinal cord and instead prematurely stop in the intermediate region of the spinal cord. As a consequence, monosynaptic connection to α-motor neurons (purple) are not established.

Er81 belongs to the Pea3 (polyoma enhancer activator 3) subfamily of ETS transcription factors comprising only three subfamily members, namely Pea3, Er81 and ets related molecule Pea3-like (Erm). These three subfamily members share a high degree of sequence identity of over 95% within their carboxy-terminal (C-terminal) ETS DNA binding domain, which is a variant of the winged helix-turn-helix motif characterized by

two α-helices separated by a tight turn and an extended loop ("wing") between to adjacent β-strands also contacting the DNA (Sharrocks, 2001; Oikawa and Yamada, 2003). The conserved ETS domain of about 85 amino acids (aa) length mediates binding to a purine-rich DNA recognition sequence containing a central 5'-GGAA/T-3' core consensus sequence flanked on either side by less well defined bases (Mo et al., 1998). Hence, specificity in target gene promoter recognition needs to be achieved by a complex regulatory network modulated at several inter- and intramolecular levels via distinct protein-protein interactions with various other transcriptional co-regulatory protein partners present within the same cell at defined times and conditions as well as through specific protein modifications such as phosphorylation and acetylation (Li et al., 2000; Verger and Duterque-Coquillaud, 2002). In addition, the majority of ETS transcription factors have been shown to be direct targets of several signal transduction pathways such as the Ras mitogen activated protein kinase (MAPK) signaling pathway (Wasylyk et al., 1998; Yordy and Muise-Helmericks, 2000). Generally, ETS transcription factors are involved in many important steps of cellular differentiation and cell lineage determination during development as well as in the adult organism (Maroulakou and Bowe, 2000; Shepherd and Hassell, 2001).

While Er81 is expressed by all proprioceptive sensory neurons from about E13 onwards, Pea3 is selectively expressed in a subpopulation of proprioceptive neurons, which has not been characterized in detail (Arber et al., 2000). Both, Er81 and Pea3 are not only expressed in proprioceptive DRG neurons but are also expressed in defined, non-overlapping pools of spinal α-motor neurons in the LMC (Lin et al., 1998; Arber et al., 2000). Moreover, both transcription factors are also present in intrafusal muscle fibers of muscle spindles, another important element of the monosynaptic stretch reflex circuit. While Pea3 is already detectable at E15.5 in intrafusal muscle fibers and thus is amongst the earliest genes expressed within differentiating muscle spindles, expression of Er81 only starts at E18, when the basic circuitry of the monosynaptic stretch reflex has already been assembled. Since many muscle spindles degenerate postnatally in *Er81*$^{-/-}$ animals, it has been suggested that Er81 might be important for muscle spindle maintenance. However, since Er81 is also required within proprioceptive neurons themselves, the

spindle phenotype in *Er81*$^{-/-}$ mice is difficult to interpret and awaits further analysis of conditional *Er81* mutants in which *Er81* gene function is eliminated only within skeletal muscles.

Erm, the third member of the Pea3 subfamily of ETS transcription factors, is also expressed in various areas of the nervous system, but not in subsets of spinal α-motor neurons (Lin et al., 1998; Hagedorn et al., 2000). In contrast to Er81 and Pea3, which in peripheral DRG are both exclusively expressed by sensory neurons, Erm expression within the DRG is not restricted to sensory neurons but also found in multipotent progenitors and glia fibrillary acidic protein (GFAP) expressing satellite glia, however not in neural crest derived Schwann cells (Hagedorn et al., 2000; Paratore et al., 2002). The exact function of Erm within these different neuronal and glial lineages still needs to be determined, as homozygous deletion of *Erm* gene function causes early embryonic lethality precluding a detailed analysis of its function within the developing nervous system.

## TOPIC OF THIS THESIS

The aim of this thesis was to characterize specific aspects of the molecular pathways controlling proprioceptive DRG sensory neuron specification during development of the mouse embryo. Two important steps during proprioceptive sensory neuron differentiation were addressed in detail.

First, the induction of expression of the ETS transcription factor Er81 in proprioceptive DRG sensory neurons by peripheral signals was analyzed. In a collaborative study the peripheral signal for the onset of Er81 protein expression was identified as muscle derived neurotrophin 3 (NT3), the ligand for the neurotrophin receptor TrkC. By combining deletion of the *NT3* gene with deletion of the pro-apoptotic Bcl2 family member gene *Bax* the early essential requirement for NT3 in mediating survival of proprioceptive DRG neurons was circumvented (White et al., 1998), thus making it possible to address later functions of NT3 during proprioceptive neuron specification. In the broader context of target induced gene expression, reviewed in the next chapter of this thesis, these findings can be considered as important examples for a general principle of

how pre- and postsynaptic partner neurons are matched with respect to their target field size and their correct functional connectivity during development.

The second major part of this thesis focuses on the early developmental function of the runt related transcription factor Runx3 in proprioceptive DRG neuron specification. Runx3 has recently been found to be required for maintenance of TrkC expression and/or survival of proprioceptive DRG neurons as well as for correct guidance of proprioceptive axons to their central targets (Inoue et al., 2002; Levanon et al., 2002).
By generating conditional gain-of-function mouse mutants allowing spatially and temporally specific misexpression of Runx3 in all DRG sensory neurons soon after their generation we found that Runx3 is required for the segregation of neurotrophic factor receptor phenotypes within early DRG sensory neurons. In absence of Runx3 function, proprioceptive sensory neurons are not progressing to a TrkC single positive state but instead maintain a TrkB/TrkC hybrid neuronal phenotype. These findings provide genetic evidence that Runx3 contributes to proprioceptive sensory neuron specification by coordinately preventing the acquisition of an alternative mechanoreceptive sensory neuron fate in early TrkC expressing proprioceptive DRG neurons through extinction of a concurrent TrkB phenotype.

# CHAPTER 2

## CONTROL OF NEURONAL PHENOTYPE: WHAT TARGETS TELL THE CELL BODIES

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## 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.

## 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).

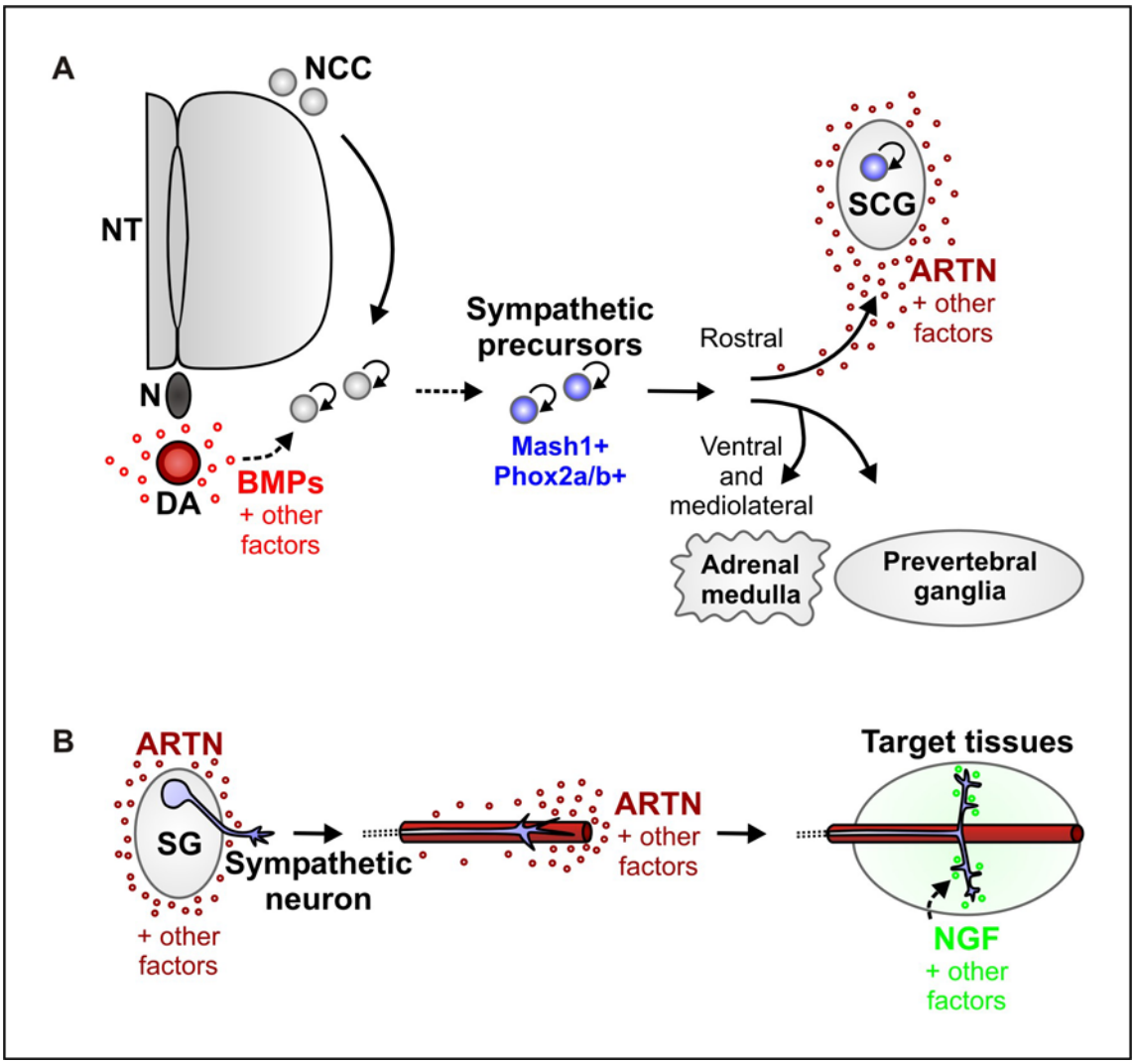


**Figure 3.** Target-derived signals and the differentiation of sympathetic neurons

**(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. Abbreviations: N=notochord; SG= sympathetic ganglia.

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 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 Bcl2 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; Livet et al., 2002; Arber et al., 2000). 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 neuron 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 neuron 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.

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).

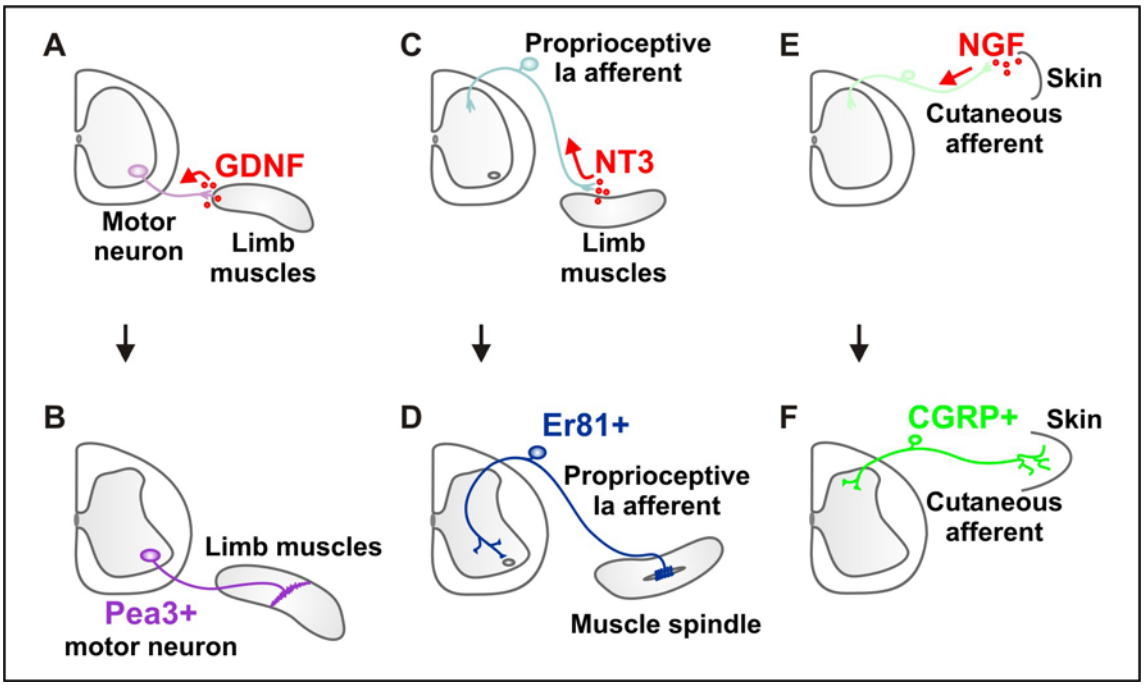


**Figure 4.** Peripheral signals control the formation of DRG sensory and motor neuron projections

**(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 neuron pool clustering in the spinal cord (B). **(C, D)** Proprioceptive Ia afferents extend two axonal branches. The peripheral branch is exposed to target-derived NT3 (C). NT3 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 calcitonin gene-related peptide (CGRP; F).

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 NT3 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 NT3 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/NT3* 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/NT3* 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 NT3 may also control the induction or activation of transcription factors other than Er81 within proprioceptive afferents. In addition to the role of NT3 in the induction of Er81 during neuronal circuit assembly, muscle spindle derived NT3 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., 2002).

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).

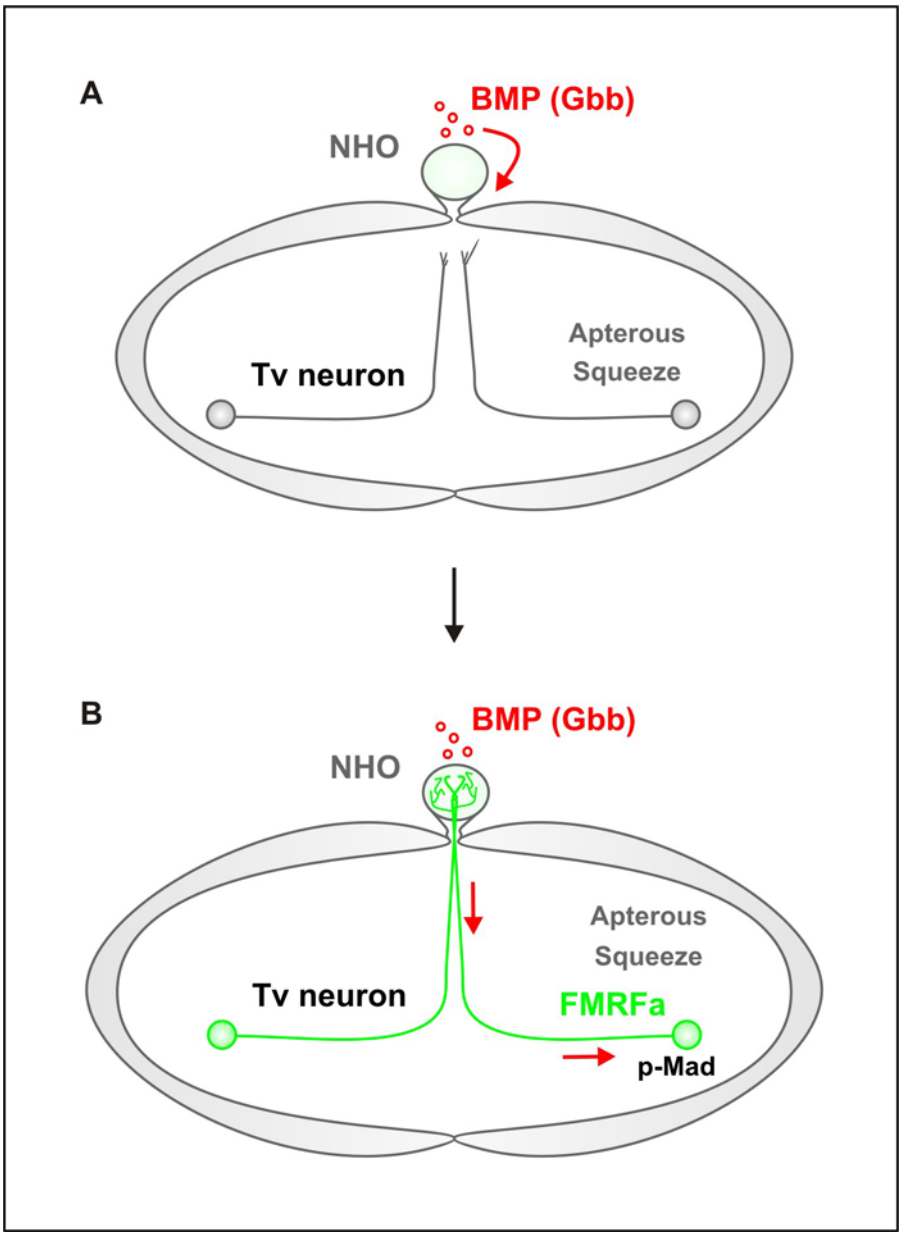


**Figure 5.** BMP signaling controls peptidergic neuronal differentiation in *Drosophila*

**(A)** 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). In the vicinity of the NHO, BMP (Glass bottom boat, or Gbb) signals are encountered. **(B)** BMP 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 the Smad homologue Mothers against decapentaplecgic (Mad, 'p' indicates phosphorylation). Transcriptional responses downstream of p-Mad, in combination with the presence of Apterous and Squeeze, result in the upregulation of the neuropeptide FMRFamide (FMRFa) within Tv neurons.

These neurons express a characteristic combination of transcription factors and the neuropeptide FMRFamide (FMRFa; Allan et al., 2003; Marques et al., 2003; Nichols, 2003; Benveniste et al., 1998). 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 (Allan et al., 2003; Benveniste et al., 1998). These findings argue for a permissive rather than an instructive role of BMP signaling in FMRFa induction 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 an 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 NT3 (Patel et al., 2003).

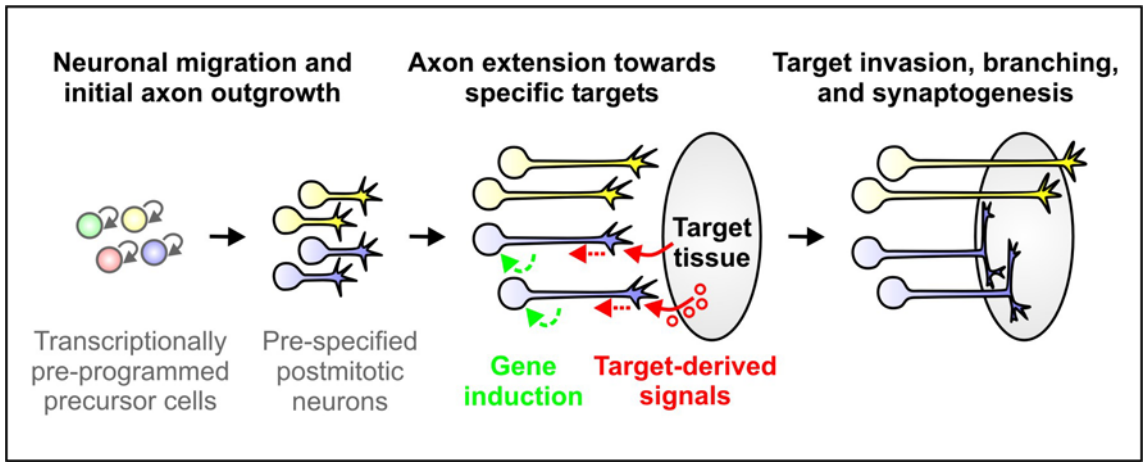


**Figure 6.** Control of neuronal phenotype by target-derived factors

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.

In *Drosophila*, the expression of FMRFa in Tv neurons not only requires target-derived 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 (Figure 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.

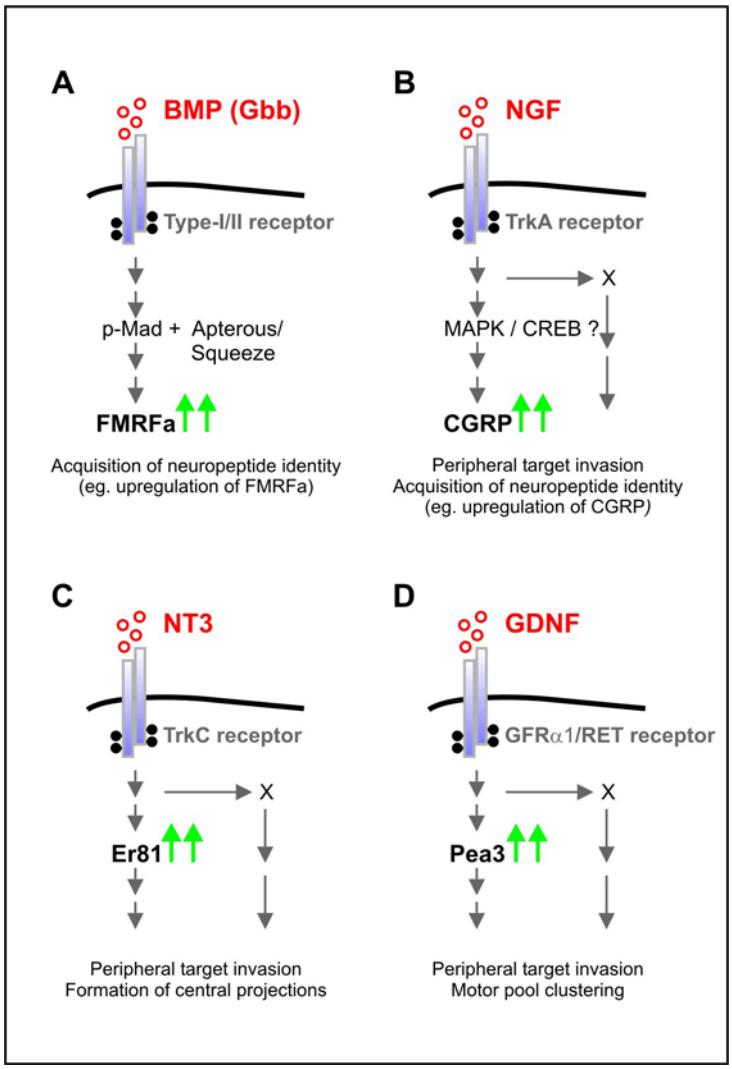


**Figure 7.** Intracellular signaling in response to target-derived factors

**(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 NT3 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 NT3 (X) might be required for proper peripheral target invasion. **(D)** GDNF binding to GFRα1/Ret receptor complexes leads to upregulation 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 neuron pool clustering.

# CHAPTER 3

# PERIPHERAL NT3 SIGNALING IS REQUIRED FOR ETS PROTEIN EXPRESSION AND CENTRAL PATTERNING OF PROPRIOCEPTIVE SENSORY AFFERENTS

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## SUMMARY

To study the role of NT3 in directing axonal projections of proprioceptive dorsal root ganglion (DRG) neurons, *NT3*$^{-/-}$ mice were crossed with mice carrying a targeted deletion of the pro-apoptotic gene *Bax*. In *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, NT3 dependent neurons survived and expressed the proprioceptive neuronal marker parvalbumin. Initial extension and collateralization of proprioceptive axons into the spinal cord occurred normally, but proprioceptive axons extended only as far as the intermediate spinal cord. This projection defect is similar to the defect in mice lacking the ETS transcription factor Er81 (Arber et al., 2000). Few if any DRG neurons from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice expressed Er81 protein. Expression of a *NT3* transgene in muscle restored DRG Er81 expression in *NT3*$^{-/-}$ mice. Finally, addition of NT3 to DRG explant cultures resulted in induction of Er81 protein. Our data indicate that NT3 mediates the formation of proprioceptive afferent-motor neuron connections via regulation of Er81.


## INTRODUCTION

Neurons of the dorsal root ganglia (DRG) are specialized to convey distinct somatic sensory modalities from the periphery to the central nervous system. Proprioceptive sensory neurons supply skeletal muscle and serve to provide information about muscle length and tension essential for coordinated motor function. Peripherally, group Ia and II proprioceptive neurons terminate on muscle spindles, whereas group Ib afferents innervate Golgi tendon receptors (Zelena, 1994). The central branches of proprioceptors project to the spinal cord and form synaptic connections with interneurons in the intermediate zone of the spinal cord (group Ia, Ib, and II afferents) or directly with motor neurons in the ventral horn of the spinal cord (group Ia and group II afferents; Brown, 1981). The specificity of connections between proprioceptive afferents and motor neurons in the mature spinal cord is well characterized (Eccles et al., 1957; Brown, 1981), but much less is known about the factors regulating proprioceptive axon extension and targeting during development.

Members of the ETS family of transcription factors have been implicated in regulating the formation of synaptic connections between group Ia sensory afferents and motor neurons (Lin et al., 1998; Arber et al., 2000). Two ETS family members, Er81 and Pea3, are expressed by developing proprioceptive sensory neurons as well as by motor neurons in the spinal cord (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002). In the chick embryo, proprioceptive sensory neurons and motor neuron pools projecting to a given muscle exhibit the same pattern of ETS gene expression, and this coordinated expression is regulated by signals from peripheral target tissue (Lin et al., 1998; see also Haase et al., 2002). In mouse, Er81 is initially expressed by most or all proprioceptive neurons in the DRG (Arber et al., 2000). Consistent with this observation, virtually all proprioceptive neurons fail to establish direct monosynaptic connections with motor neurons of *Er81*$^{-/-}$ mice, and terminate instead in the intermediate zone of the spinal cord (Arber et al., 2000). Since proprioceptive neuronal survival is not affected in *Er81*$^{-/-}$ mice, Er81 expression is likely to regulate the expression of molecules necessary for the establishment of the appropriate terminal arborization of group Ia and II afferents within the ventral spinal cord (Arber et al., 2000). Although interactions with the periphery appear to be important in the regulation of Er81 expression (Lin et al., 1998), the identity of the relevant inductive factor(s) responsible for this regulation is unknown.

The two major functional classes of DRG neurons, proprioceptive and cutaneous sensory neurons, are distinguished by their expression of different receptor tyrosine kinases (Trks) that transduce signals provided by different members of the neurotrophin family of polypeptide growth factors (for reviews see Snider, 1994; Bibel and Barde, 2000; Huang and Reichardt, 2001). Neurotrophin signaling via receptor tyrosine kinases underlies, in large part, the target dependence of peripheral neurons during critical developmental periods. Proprioceptive DRG neurons, which comprise about 20% of the adult DRG neuronal population, express TrkC and require neurotrophin 3 (NT3) signaling for their survival during development. Elimination of TrkC signaling in mice results in a 20%–35% loss of DRG neurons and the absence of central proprioceptive projections (Klein et al., 1994; Tessarollo et al., 1994; see also Ernfors et al., 1994; Farinas et al., 1994). In contrast, inactivation of NT3 in mice results in a ~70% reduction in the number of DRG

neurons and thus appears to result in an additional loss of nonproprioceptive sensory neurons (Ernfors et al., 1994; Farinas et al., 1994; Tessarollo et al., 1994).

In addition to their role in regulating neuronal survival, there is emerging evidence that neurotrophins have regulatory effects on neuronal morphology, notably functions related to axonal growth and arborization in target fields (for reviews see McAllister et al., 1999; Bibel and Barde 2000; Huang and Reichardt 2001 and Markus et al., 2002a). However, the dependence of peripheral neurons on neurotrophin signaling for survival at early developmental stages has limited our understanding of the requirements for neurotrophins in regulating late developmental events *in vivo*. Our previous work has demonstrated that genetic elimination of the Bcl2 family member *Bax*, which is required for apoptosis upon neurotrophin withdrawal (Deckwerth et al., 1996), permits TrkA expressing cutaneous DRG neurons to survive in the absence of neurotrophin signaling *in vivo* (Patel et al., 2000). These neurons extend central processes through the dorsal roots into the spinal cord, but the growth of the peripheral process in the limb is markedly impaired (Patel et al., 2000). Thus, elimination of *Bax*, in principle, might provide a tool to explore axon growth and targeting of proprioceptive neurons in the absence of NT3.

NT3 appears to play a complex role in the regulation of proprioceptive axon extension and targeting. During development, NT3 is expressed by skeletal muscle, by mesenchyme surrounding peripheral projection pathways and by motor neurons in the spinal cord (Schecterson and Bothwell 1992; Ernfors et al., 1992; Patapoutian et al., 1999). Injection of blocking anti-NT3 antibodies into the limb during the period of naturally occurring cell death results in a decrease in the number of proprioceptive neurons (Oakley et al., 1995), indicating that NT3 derived from peripheral tissues is required for the survival of proprioceptive neurons. Furthermore, developing peripheral sensory axons can be directed to grow toward local sources of NT3 and other neurotrophins (Tucker et al., 2001), raising the possibility that endogenous NT3 might play a role in supporting interstitial axon extension in the developing limb. It also seems plausible that NT3 derived from either peripheral or spinal cord sources might influence central proprioceptive axon projections. However, it remains to be established whether

NT3 regulates the collateralization and targeting of central proprioceptive axons to motor neurons, and if so, by what mechanism.

In order to assess the role of NT3 on proprioceptive axon extension and Er81 expression, we crossed mice carrying a targeted deletion of the *Bax* gene with *NT3*$^{-/-}$ mice. We report here that proprioceptive sensory neurons survive in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. However, peripheral proprioceptive axons and their associated muscle spindles are absent at birth in these mice. Furthermore, although the initial collateralization of central proprioceptive axons into the spinal cord proceeds normally in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, these axons do not project toward motor neurons and instead terminate in the intermediate spinal cord, a phenotype similar to that observed in *Er81*$^{-/-}$ mice (Arber et al., 2000). Consistent with this observation, we find that DRG neurons from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice express markedly reduced levels of Er81 protein, and we show that exogenous NT3 can induce the expression of Er81 in proprioceptive neurons of DRG explant cultures. Furthermore, we provide evidence that a peripheral rather than a central source of NT3 is required for Er81 expression and central targeting of group Ia and II afferents toward motor neurons.

# RESULTS

## ELIMINATION OF BAX RESTORES DRG NEURONAL NUMBER IN THE ABSENCE OF NT3/TRK SIGNALING

DRG neurons survive in the absence of neurotrophin signaling *in vivo* if *Bax* is also deleted (Patel et al., 2000). To study the effects of NT3 on the development of proprioceptive sensory neurons, *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice were generated by crossing heterozygotes from each line. At birth, *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice were overtly indistinguishable from their wild-type littermates. However, *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice did not survive beyond the first postnatal week, as is the case with *NT3*$^{-/-}$ mice (Ernfors et al., 1994).

We assessed peripheral sensory neuron survival in the progeny of *Bax/NT3* crosses by counting lumbar level 4 (L4) DRG neurons in semithin sections at P0 (Figure 8A). As

expected, there was a ~70% loss of DRG neurons in $Bax^{+/+}/NT3^{-/-}$ mice compared to wild-type. Strikingly, no neuronal loss occurred in the L4 DRG of $Bax^{-/-}/NT3^{-/-}$ mice. Rather, we found a ~50% increase in the total number of DRG neurons in the $Bax^{-/-}/NT3^{-/-}$ mice (12,932 ± 758), comparable to that found in $Bax^{-/-}/NT3^{+/+}$ mice (14,160 ± 1013).

**A DRG neuron and dorsal root axon counts at P0**

| Genotype | $Bax^{+/+}$, $NT3^{+/+}$ | $Bax^{-/-}$, $NT3^{+/+}$ | $Bax^{+/+}$, $NT3^{-/-}$ | $Bax^{-/-}$, $NT3^{-/-}$ |
|---|---|---|---|---|
| L4 DRG neuron counts | 8618 ± 483 (100%) | 14,160 ± 1013 (164%) | 2531 (30%, n= 2) | 12 932 ± 758 (150%) |
| L4 dorsal root axons | 8800 | 13,003 | 2603 (n = 2) | 14,485 |

n = 5 for DRG neuron counts; n = 3 for dorsal root axon counts

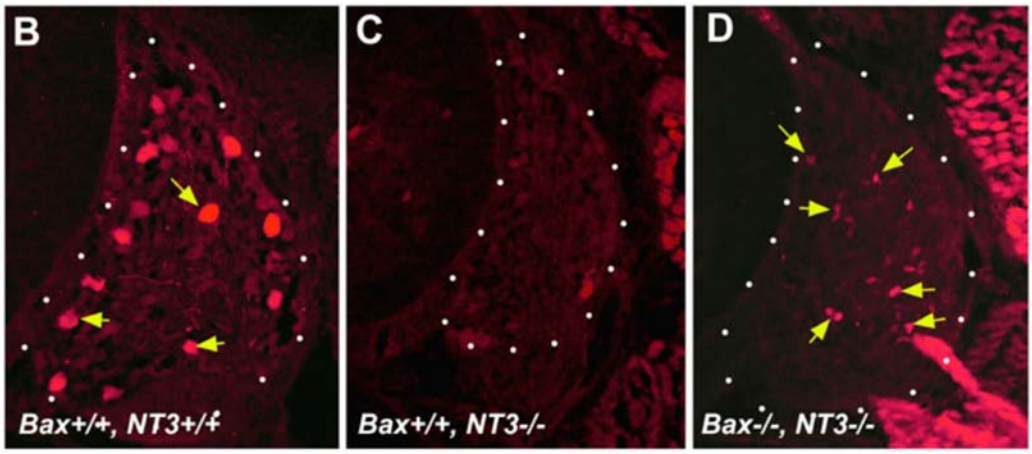


**Figure 8.** Proprioceptive DRG neurons survive in $Bax^{-/-}/NT3^{-/-}$ mice

**(A)** L4 DRG neuron and L4 dorsal root axon counts at P0. There is no significant difference in the number of DRG neurons between $Bax^{-/-}/NT3^{+/+}$ (14,160 ± 1013) and $Bax^{-/-}/NT3^{-/-}$ (12,932 ± 758) mice. These values represent a 64% and 50% increase, respectively, over wild-type (8618 ± 483). Note that the number of dorsal root axons corresponds well with the number of DRG neurons for each genotype, demonstrating that the additional DRG neurons in $Bax^{-/-}/NT3^{+/+}$ and $Bax^{-/-}/NT3^{-/-}$ mice extend their central axons through the dorsal roots toward the spinal cord.
**(B–D)** Parvalbumin immunohistochemistry in P0 DRG sections. In wild-type DRG (B), parvalbumin expression is found in large neurons (arrows), consistent with expression by proprioceptive neurons. As expected, parvalbumin immunoreactivity is virtually absent in DRG sections from the $Bax^{+/+}/NT3^{-/-}$mice (C), consistent with the complete loss of proprioceptive neurons in these mice. In contrast, numerous parvalbumin+ neurons can be seen in DRG sections from $Bax^{-/-}/NT3^{-/-}$ mice (D; arrows). Note, however, that these rescued parvalbumin+ neurons are considerably smaller than parvalbumin+ neurons in wild-type DRG.

These findings indicate that the elimination of Bax restores neuronal number in the DRG in the absence of NT3. The supranormal number of neurons in both $Bax^{-/-}/NT3^{+/+}$ and

*Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is presumably due to the absence of naturally occurring neuron death in the *Bax* null mutants (White et al., 1998; Patel et al., 2000).

To assess whether proprioceptive neurons survived in the absence of NT3 signaling, we examined DRG sections from P0 mice for expression of the calcium binding protein parvalbumin (Figure 8B–D). Parvalbumin is an established marker of NT3 dependent proprioceptive neurons (Copray et al., 1994; Ernfors et al., 1994; Honda, 1995). We found parvalbumin immunoreactivity in large-diameter DRG neurons of wild-type mice (Figure 8B), and consistent with the loss of proprioceptive neurons in *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice, the number of parvalbumin expressing neurons in the DRG was dramatically reduced (Figure 8C). In contrast, we found numerous parvalbumin expressing neurons in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, demonstrating that proprioceptive neurons had survived NT3 deprivation in the absence of *Bax*. However, the cross-sectional area of parvalbumin positive DRG neuronal somata from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice was reduced by about 70% as compared to wild-type mice at P0, presumably due to the sustained loss of NT3 trophic support (Figure 8D). Further evidence that proprioceptive neurons survived until P0 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is provided by analysis of their axonal projections into the spinal cord (see below).

## A PERIPHERAL DEFECT IN PROPRIOCEPTIVE AXON PROJECTIONS IN *BAX*$^{-/-}$/*NT3*$^{-/-}$ MICE

To determine in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice whether the peripheral axons of proprioceptive neurons innervate muscles in the absence of NT3 signaling, we searched for the presence of parvalbumin positive axons in the soleus nerve at P0 (Figure 9). Parvalbumin positive axons were found to extend through the soleus nerve and penetrated the soleus muscle in P0 wild-type (*Bax*$^{+/+}$/*NT3*$^{+/+}$) mice (Figure 9B). In contrast, in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (Figure 9C), no parvalbumin positive axons were detected in the soleus nerve at P0, despite the fact that parvalbumin positive axons could be detected in the spinal cord (see below). Because NT3 could, in principle, regulate the levels of parvalbumin expression rather than the presence of peripheral axons themselves, we characterized further the

extent of peripheral innervation through axon counts in the soleus nerve at P0 (Figure 9A). We detected a 64% decrease in the number of axons in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice when compared with the *Bax*$^{-/-}$/*NT3*$^{+/+}$ mice even though the number of DRG neurons in the two mutants were similar (see Figure 8A). These findings indicate that proprioceptive sensory axons fail to innervate their target muscles in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice.

**A Soleus nerve axon counts and soleus muscle spindle counts**

| Genotype | *Bax*$^{+/+}$, *NT3*$^{+/+}$ | *Bax*$^{-/-}$, *NT3*$^{+/+}$ | *Bax*$^{+/+}$, *NT3*$^{-/-}$ | *Bax*$^{-/-}$, *NT3*$^{-/-}$ |
|---|---|---|---|---|
| Soleus nerve axon counts | 78 ± 1 | 126 ± 23 | 37 ± 0.6 | 7 ± 4 |
| Soleus muscle spindle counts | 11.2 | 11.5 (n=2) | 0 | 0 |

n ≥ 3 for axon and spindle counts

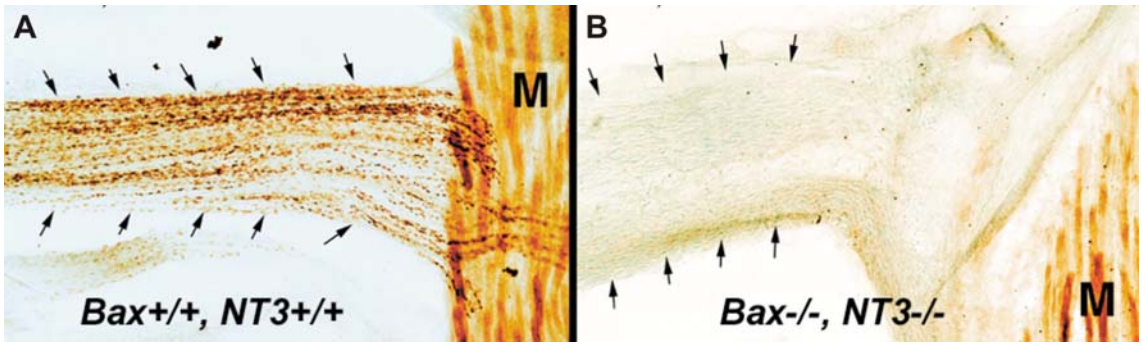


**Figure 9.** Peripheral proprioceptive axons and muscle spindles are absent at P0 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice

**(A)** Axon counts in the nerve to the soleus muscle at P0 reveal that the number of axons is reduced in *Bax*$^{-/-}$/*NT3*$^{-/-}$ (70 ± 4) mice compared to *Bax*$^{-/-}$/*NT3*$^{+/+}$ (126 ± 23), even though the number of DRG neurons is equivalent in the two mutants. Furthermore, muscle spindle counts reveal the absence of soleus muscle spindles in *Bax*$^{+/+}$/*NT3*$^{-/-}$ and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice.
**(B, C)** Parvalbumin+ axons can be seen in the nerve to the soleus muscle (outlined by arrows) from wild-type P0 mice. These axons extend through the nerve and penetrate the muscle (M) in the wild-type controls. However, in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, parvalbumin immunoreactivity is absent in the nerve to the soleus.

Muscle spindles are peripheral end organs innervated by proprioceptive neurons, and their development and maintenance are regulated by contacts of group Ia and II afferents with myotubes (Kucera and Walro, 1987; Kucera and Walro, 1988). The morphology and number of muscle spindles in the soleus muscle were identical in *Bax*$^{-/-}$/*NT3*$^{+/+}$ and wild-type mice (Figure 9A). In contrast, we found no muscle spindles in soleus muscles of *Bax*$^{+/+}$/*NT3*$^{-/-}$ or *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice at P0 (Figure 9A). Collectively, the failure to detect

parvalbumin positive axons in the soleus nerve, the deficiency in axon number, and the absence of muscle spindles in the soleus muscle demonstrate a developmental failure of proprioceptive neurons to innervate their peripheral muscle targets or an early retraction of these axonal processes.

To distinguish between these two possibilities, we assessed the development of peripheral proprioceptive projections in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos at E15 and E17. E15 represents the earliest stage at which muscle spindle formation in the mouse embryo can be detected, as assessed by the expression of the zinc finger transcription factor Egr3 and the ETS protein Pea3 (Tourtellotte et al., 2001; Hippenmeyer et al., 2002). At E15, we detected Egr3 immunoreactivity in the distal hindlimb muscles of wild-type embryos in approximately 15% of the longitudinal sections examined (n = 3, data not shown). In contrast, in E15 *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we did not detect Egr3 immunoreactivity in distal hindlimb muscles in any of the sections examined (n = 3). At E17, Egr3 is robustly expressed by intrafusal muscle fibers in wild-type embryos, and clusters of Egr3 positive fibers could be detected in roughly 15% of the sections through both forelimb and hindlimb muscles (arrows in Figure 10A–C, E). In contrast, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos analyzed at E17, no Egr3 positive fibers were detected in distal forelimb and hindlimb (n = 3; Figure 10D, F).

As in P0 mice, no parvalbumin positive axons were detected in hindlimb nerves of *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos at E15 or E17 (data not shown). To assess whether the expression of parvalbumin may not reveal the full extent of peripheral projections in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we used DiI crystals applied to sciatic nerves as an independent means of tracing peripheral projections. In wild-type embryos, this method readily revealed annulospiral endings characteristic of spindle afferents in soleus and plantaris muscles at E17 (Figure 10G, arrows). Interestingly, some axons with similar morphology were labeled in soleus and plantaris muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, analyzed at the same age (Figure 10H, I). These axons were invariably in close proximity to the main nerve trunk and did not extend to lateral aspects of the muscle. Since no parvalbumin or Egr3 expression could be detected in these preparations, our results do not resolve definitively whether these axons correspond to proprioceptive afferents. Nevertheless, these findings

reveal an embryonic defect in the development of peripheral projections of proprioceptive afferents and a corresponding defect in the initiation of muscle spindle differentiation by these proprioceptive afferents.

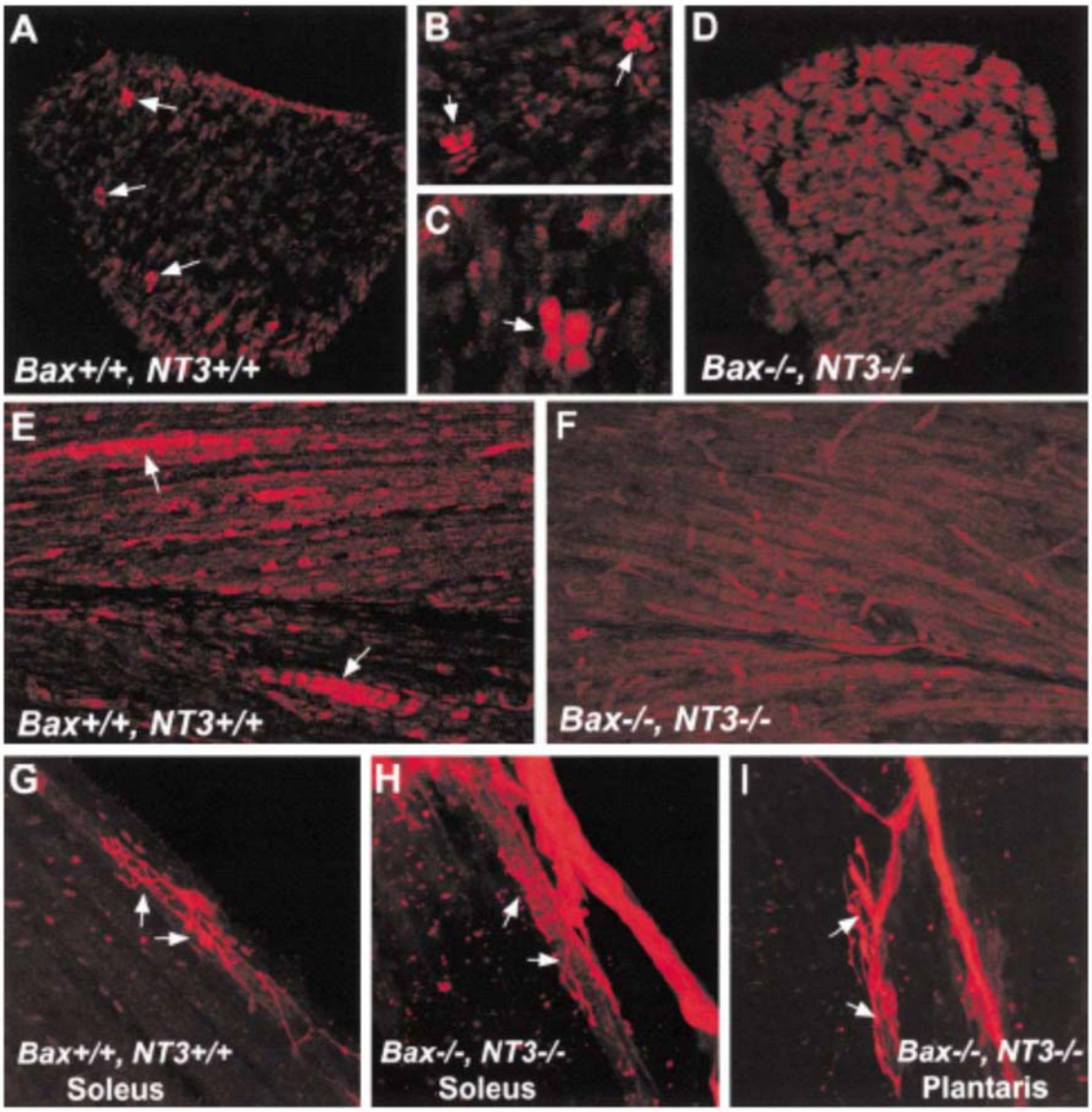


**Figure 10**. Muscle spindles do not develop in the absence of NT3 signaling in *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice

**(A–F)** Egr3 immunoreactivity in distal hindlimb and forelimb skeletal muscle. In cross-sections through wild-type soleus muscle (A–C) and longitudinal sections through the distal forelimb (E), Egr3 immunoreactivity is present in intrafusal muscle fibers at E17. Egr3 immunoreactivity is absent in hindlimb (soleus in D) and forelimb (F) muscles in *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice. **(G–I)** DiI-labeled peripheral axons in distal hindlimb muscles at E17. The endings of proprioceptive afferents exhibit a characteristic annulospiral structure in the wild-type soleus muscle (G; arrow). A few spiral-like axonal endings are found in the soleus and plantaris muscles from *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice (H, I; arrows). In contrast to controls, such endings in *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice are invariably in close proximity to the major nerve trunk.

## PROPRIOCEPTIVE AXONS FAIL TO EXTEND INTO THE VENTRAL HORN IN *BAX*$^{-/-}$/*NT3*$^{-/-}$ MICE

The growth of the central and peripheral axon branches of TrkA expressing DRG neurons is differentially regulated by NGF signaling (Patel et al., 2000). In order to assess whether proprioceptive neurons extend their axons centrally into the spinal cord in the absence of NT3 signaling, we counted dorsal root axons at P0 by sampling electron micrographs of lumbar dorsal root sections (Figure 8A). Across all genotypes, there was a tight correspondence between DRG neuronal number and dorsal root axon counts, indicating that surviving neurons in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos do extend their central processes into the dorsal roots, toward the spinal cord (Figure 8A).

To characterize further the central projections of proprioceptive afferents, we traced the axons into the spinal cord at E15, by DiI labeling (Figure 11). By E15, proprioceptive afferents have started to invade the ventral horn at all levels of the spinal cord in wild-type mice (Figure 11A). In *Bax*$^{+/+}$/*NT3*$^{-/-}$ embryos, DiI-labeled axons projected only into the dorsal laminae of the spinal cord, consistent with the early death of proprioceptive neurons observed in the absence of NT3 (Figure 11B). In contrast, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, proprioceptive axons entered the spinal cord and followed a trajectory similar to that in wild-type controls (n = 4; Figure 11C, arrows). However, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, proprioceptive axons stopped in the intermediate zone of the spinal cord and failed to project toward motor neurons in the ventral horn (Figure 11, yellow asterisks). Parvalbumin immunostaining in *Bax*$^{-/-}$/*NT3*$^{+/+}$ revealed the presence of proprioceptive afferents in the ventral horn of the spinal cord, indicating that the projection defect observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is due to the absence of NT3 rather than the absence of Bax (data not shown). In order to quantify DiI-labeled proprioceptive afferents, we measured the fluorescence intensity of DiI-labeled afferents in *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Bax*$^{+/+}$/*NT3*$^{+/+}$ mice at E15. The mean fluorescence intensity measurements of DiI-labeled afferents in the intermediate zone of *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice were comparable to the mean density measurements of DiI-labeled afferents in the ventral horn of *Bax*$^{+/+}$/*NT3*$^{+/+}$ mice

with a ratio of 1.02 ($Bax^{-/-}/NT3^{-/-}$ versus $Bax^{+/+}/NT3^{+/+}$), indicating that the overall number of proprioceptive collaterals was roughly comparable in the two mutants.

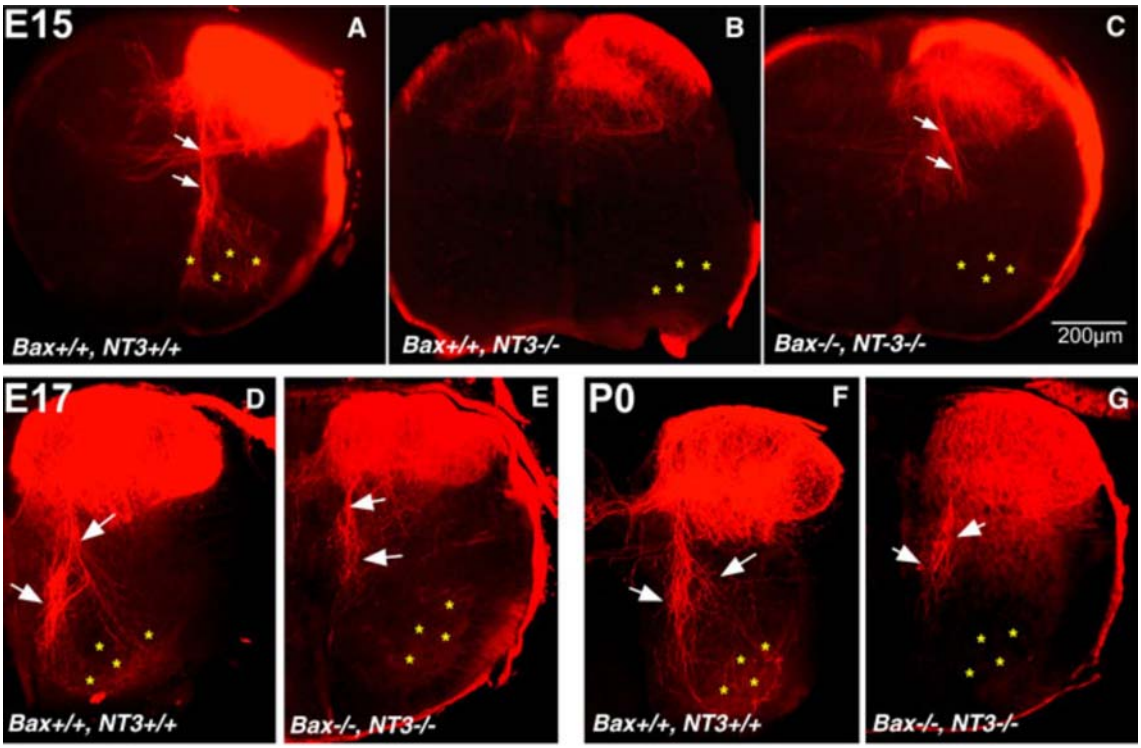


**Figure 11.** Central proprioceptive projections do not reach the ventral spinal cord in $Bax^{-/-}/NT3^{-/-}$ mice

DiI tracing of the central DRG projections at E15 (A–C), E17 (D, E), and P0 (F, G). In E15 wild-type embryos (A), proprioceptive axons (arrows) penetrate the spinal cord and project ventrally to the motor neurons in the ventral horn (yellow asterisks) of the spinal cord. As expected, the ventrally projecting proprioceptive axons are absent in the spinal cord of E15 $Bax^{+/+}/NT3^{-/-}$ mice (B). In E15 $Bax^{-/-}/NT3^{-/-}$ mice (C), the axons (arrows) of the rescued proprioceptive neurons penetrate the spinal cord but extend only as far as the intermediate spinal cord and do not project to the motor neurons in the ventral horn (yellow asterisks). DiI-labeled central projections at E17 (D, E) and P0 (F, G) reveal that the proprioceptive axons of $Bax^{-/-}/NT3^{-/-}$ mice never innervate the motor neurons in the ventral horn of the spinal cord.

To examine whether axonal projections into the ventral spinal cord were simply delayed developmentally, we traced central projections with DiI at E17 (Figure 11D, E) and P0 (Figure 11F, G). No afferent projections were detected in the ventral horn at either of these ages in $Bax^{-/-}/NT3^{-/-}$ embryos (E17, n = 3; P0, n = 2). Parvalbumin staining at P0 verified that these axons were indeed from proprioceptive afferents (n = 3, data not shown).

## PROPRIOCEPTIVE DRG NEURONS SHOW REDUCED ER81 EXPRESSION IN THE ABSENCE OF NT3 SIGNALING

A defect in the central projections of proprioceptive afferents similar to that in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice occurs in mice lacking the ETS transcription factor Er81 (Arber et al., 2000). The failure of proprioceptive axons to project into the ventral horn in both *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Er81*$^{-/-}$ mutants raised the possibility that the central projection defect observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is mediated through regulation of Er81 expression. The subset of DRG neurons that express Er81 coexpress TrkC and parvalbumin and correspond to proprioceptive afferents (Arber et al., 2000). We examined whether proprioceptive DRG neurons expressed Er81 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Unlike at P0, the cross-sectional area of parvalbumin positive DRG neurons in wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice was equivalent at E15 (781 ± 37 μm$^2$ in wild-type and 727 ± 32 μm$^2$ in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos; Figure 12A–C). The intensity of parvalbumin staining, however, appeared to be fainter in DRG from the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice than wild-type. At E15, numerous Er81 positive neurons were detected in DRG from wild-type (Figure 12E) and *Bax*$^{-/-}$/*NT3*$^{+/+}$ mice (not shown), but no Er81 protein expression was detected in DRG sections from *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos (n = 4; Figure 12G). Er81 expression was also not detected in DRG of *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice, but this reflects the loss of all proprioceptive neurons (Figure 12F).

To assess whether there was a similar reduction in *Er81* mRNA in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we examined *Er81* mRNA levels in E15 lumbar level DRG by *in situ* hybridization (n = 2; Figure 12I–K). Compared to wild-type, we observed a marked reduction in the number of DRG neurons expressing *Er81* mRNA in DRG from *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos. In addition, the intensity of the *Er81* mRNA labeling in individual neurons was reduced in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ DRG compared to wild-type.

The effect of NT3 on Er81 expression appears to be selective to DRG neurons. Subsets of motor neurons in the ventral horn of the spinal cord also express Er81 (Arber et al., 2000). At E15, *Er81* mRNA and Er81 protein (Figure 12L–O) were expressed in similar patterns in lumbar regions of the spinal cord in wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos.

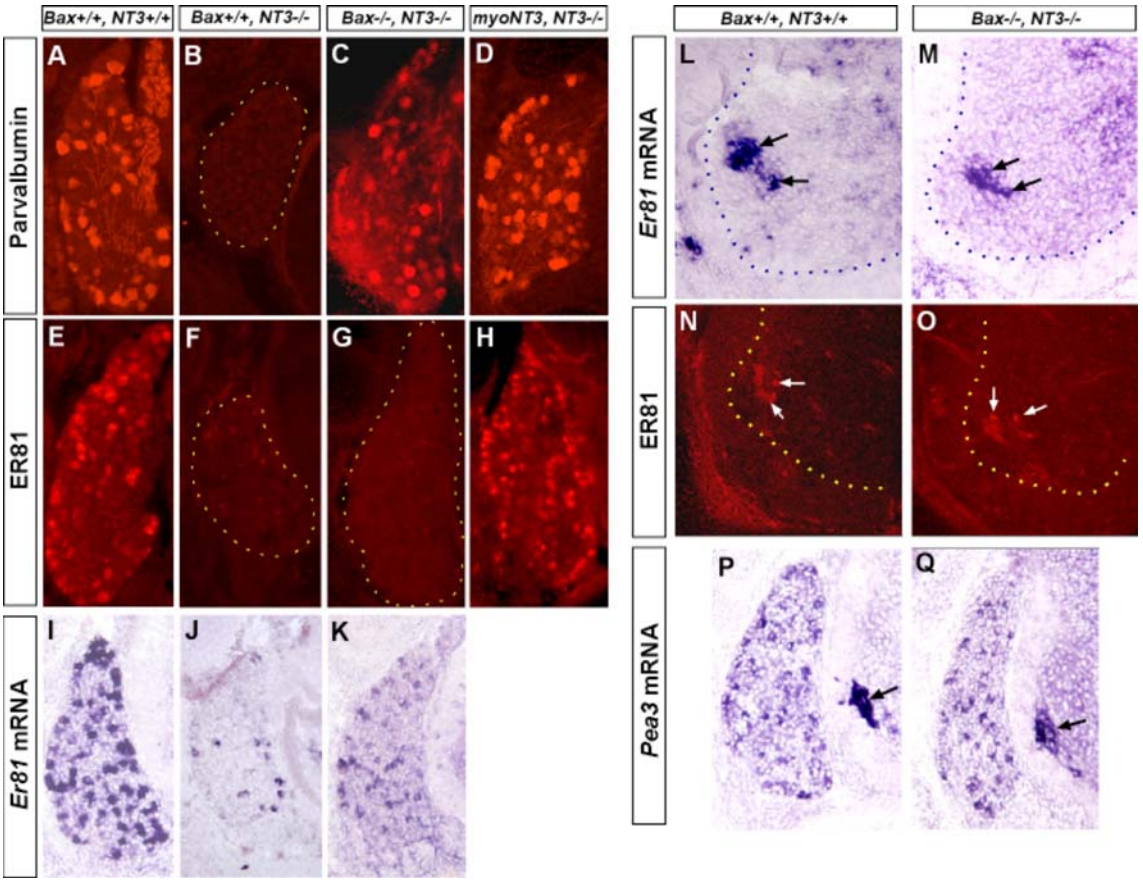


**Figure 12.** Proprioceptive DRG neurons do not express Er81 in the absence of NT3 signaling

**(A–D)** Parvalbumin+ neurons are found in lumbar DRG sections from E15 wild-type and *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice. As expected, parvalbumin+ neurons are not found in DRG sections from *Bax*$^{+/+}$*/NT3*$^{-/-}$ mice. Parvalbumin expressing neurons are also rescued in *myoNT3/NT3*$^{-/-}$ mice. **(E–H)** Numerous Er81 expressing neurons are present in the wild-type E15 DRG. In contrast, Er81 immunoreactivity is absent in DRG sections from the E15 *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice even though the proprioceptive neurons survive and express parvalbumin in these mice. Er81 expression is rescued in DRG neurons from *myoNT3/NT3*$^{-/-}$ mice. **(I–K)** Consistent with the absence of Er81 immunoreactivity in *Bax*$^{-/-}$*/NT3*$^{-/-}$ DRG neurons, there is also a marked reduction in *Er81* mRNA levels as revealed by *in situ* hybridization. However, mRNA expression is not abolished in *Bax*$^{-/-}$*/NT3*$^{-/-}$ DRG neurons. **(L–O)** *In situ* hybridization and immunohistochemistry reveal that expression of *Er81* mRNA (L and M) and protein (N, O) in a subset of spinal motor neurons (arrows) is unaffected in *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice (dots demarcate the boundary of the ventral horn). **(P, Q)** *In situ* hybridization reveals no qualitative difference in pattern or levels *Pea3* mRNA expression between DRG from E15.5 wild-type and *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice. Also note that *Pea3* mRNA is present in motor neurons of both wild-type and *Bax*$^{-/-}$*/NT3*$^{-/-}$ mice (arrows).

In addition, to assess whether the regulation of ETS genes by NT3 is restricted to Er81, we assessed the expression of Pea3, a member of the ETS gene family closely related to Er81, in DRG neurons of *Bax*$^{-/-}$*/NT3*$^{-/-}$ embryos. Pea3 is expressed by both a subset of proprioceptive afferents and a subset of cutaneous DRG neurons (Arber et al., 2000).

There was no noticeable difference in patterns or levels of *Pea3* mRNA expression between DRG from wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos (Figure 12P, Q). This result provides evidence for specificity of NT3 in regulating Er81 expression in DRG neurons although it does not absolutely exclude the possibility that Pea3 expression in proprioceptive but not cutaneous sensory neurons is affected.

## NT3 INDUCES ER81 EXPRESSION IN PROPRIOCEPTIVE DRG NEURONS

The absence of Er81 expression in DRG from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice and the similarity in the central projection phenotype with *Er81*$^{-/-}$ mice raises the possibility that NT3 is responsible for the induction of Er81 in DRG neurons. To test this hypothesis, we cultured wild-type mouse DRG *in vitro* in the presence or absence of NT3 and assessed Er81 protein expression. Since the onset of Er81 expression in DRG does not occur until E13 *in vivo* (Arber et al., 2000), DRG explants were cultured from E11.5 and E12.5 (not shown) embryos. We did not detect Er81 immunoreactivity in DRG explants cultured in the absence of NT3 at any time point up to 18 hours even though many DRG neurons expressed the LIM homeodomain protein Isl1. In the presence of NT3, induction of Er81 expression was detected in a subset of Isl1 positive neurons within 3 hours of culturing the explants, and expression was maintained for up to 18 hours *in vitro* (Figure 13). In contrast, no expression of Er81 was detected in DRG explants cultured in the presence of NGF for 18 hours (Figure 13).

To rule out the possibility that NT3 dependent DRG neurons die within a few hours of NT3 deprivation *in vitro*, we cultured E12.5 DRG explants from *Bax*$^{-/-}$ embryos for 18 hours in the presence or absence of NT3. Both NGF and NT3 dependent neurons from *Bax*$^{-/-}$ DRG neurons survive several days *in vitro* in the absence of neurotrophin signaling (Lentz et al., 1999). Consistent with the results from wild-type explants, we found numerous Er81 positive neurons in *Bax*$^{-/-}$ DRG explants cultured in the presence of NT3 for 18 hours *in vitro* (Figure 14E, F). In contrast, few if any Er81 positive neurons were found in DRG explants cultured in the absence of NT3 (Figure 14B, C).

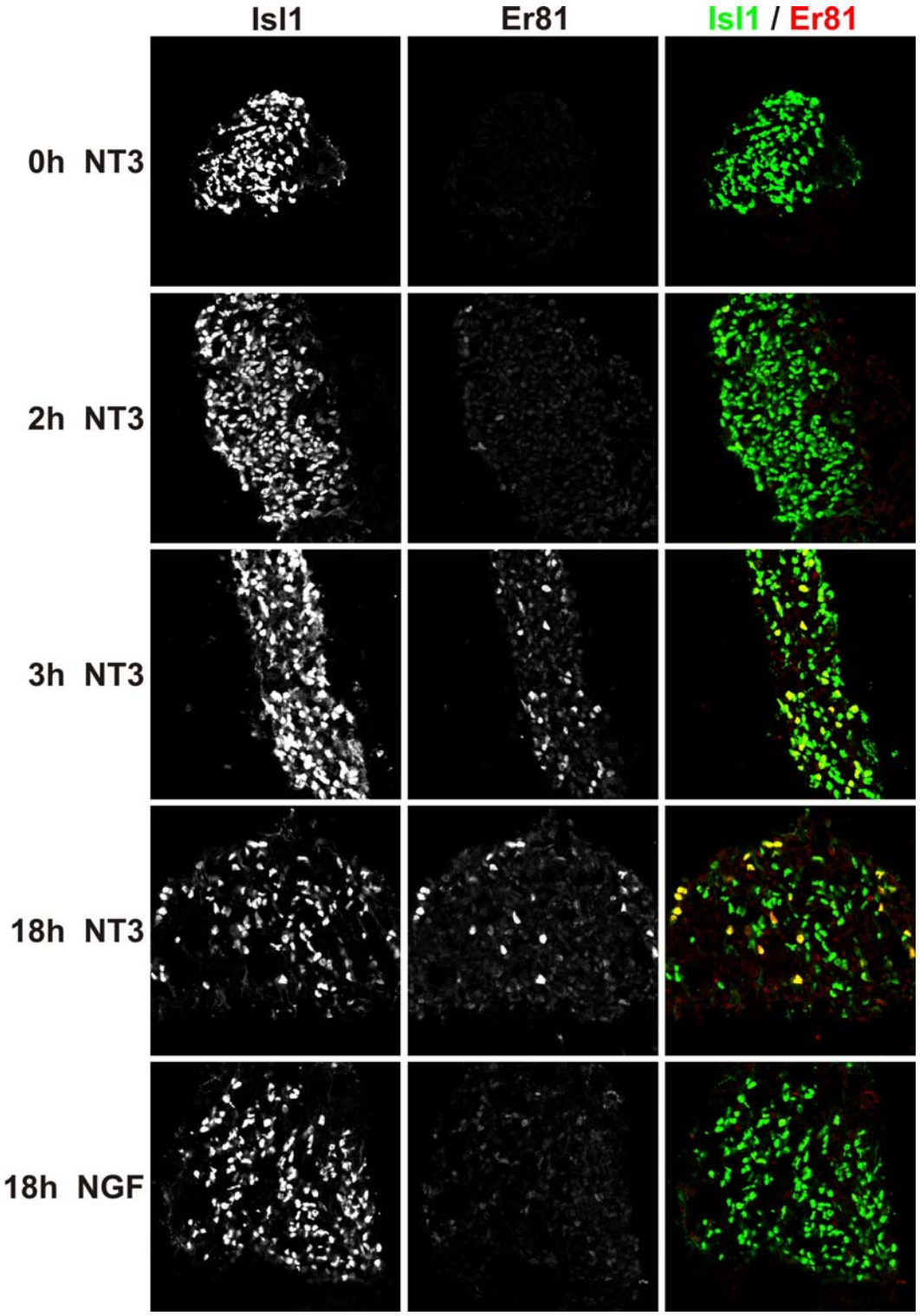


**Figure 13**. Rapid induction of Er81 expression by NT3 in E11.5 DRG explants

Er81 expression is induced in the presence of NT3 in a subset of Isl1+ DRG neurons within three hours of culturing the explants. In the presence of NGF, however, even after 18 hours *in vitro*, Er81 expression is not induced in DRG neurons.

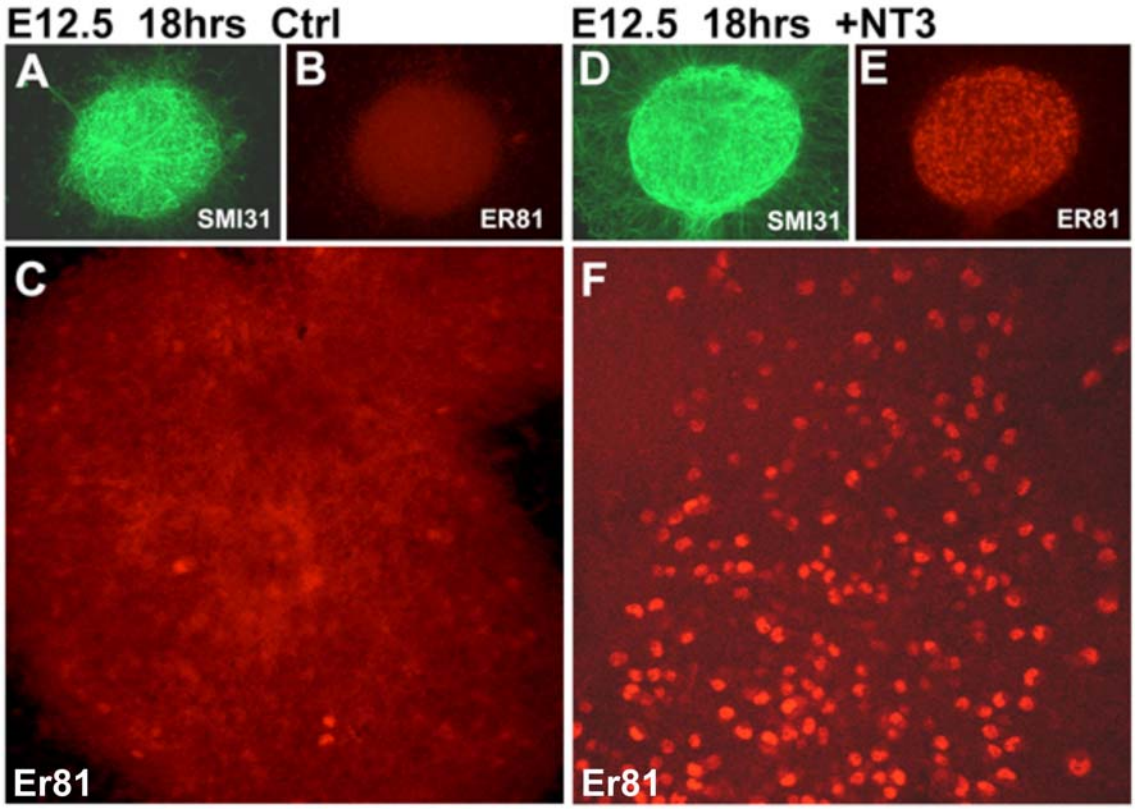


**Figure 14.** Induction of Er81 Expression in E12.5 *Bax*$^{-/-}$ DRG explants is NT3 dependent

Confocal images of neurofilament (SMI31; [A, D]) and Er81 and staining (B, E) in DRG explants from E12.5 *Bax*$^{-/-}$ mice. Er81+ neurons are found after 18 hours *in vitro* in the presence of NT3 (E, F). Few if any *Bax*$^{-/-}$ DRG neurons exhibit Er81 immunoreactivity in the absence of NT3 (B, C), even after 18 hours. Neurofilament staining demonstrates that *Bax*$^{-/-}$ DRG neurons survive *in vitro* for 18 hours even in the absence of neurotrophin signaling.

## SOURCES OF NT3 REGULATING ER81 EXPRESSION AND THE DEVELOPMENT OF CENTRAL PROJECTIONS OF PROPRIOCEPTIVE NEURONS

We next addressed the question of whether the source of NT3 responsible for regulating Er81 expression in proprioceptive neurons and the development of ventral projections by group Ia and II afferents is located peripherally or within the spinal cord.

Within the developing spinal cord, motor neurons are known to be a major source of NT3 (see Wright et al., 1997, and references therein). We therefore analyzed a mouse mutant in which motor neurons are ablated genetically by diphtheria toxin expression as soon as

they are postmitotic, and thus long before group Ia and II afferents project into the ventral spinal cord (Yang et al., 2001; Pun et al., 2002). In these mice we found normal expression of both Er81 and parvalbumin in DRG neurons at E17.5 (Figure 15A–F). Group Ia and II afferents innervated the muscles and were capable of inducing muscle spindles expressing Pea3 (Figure 15G–J). In addition, the central projections of proprioceptive afferents in these mice were not impaired in their ability to project into the ventral spinal cord (Figure 15K–N), arguing against a role for motor neuron derived NT3 in the induction of Er81 in proprioceptive afferents and the control of the central patterning of group Ia and II afferents.

Second, to assess whether peripheral NT3 is sufficient to restore the expression of Er81 in $NT3^{-/-}$ mice, we crossed $NT3^{-/-}$ mice with a strain of mice selectively overexpressing *NT3* in skeletal muscles under the control of the *myogenin* promoter (*myoNT3* mice). In *myoNT3*/$NT3^{-/-}$ mice, parvalbumin positive proprioceptive neurons are rescued from apoptotic cell death and these rescued neurons project their axons to the ventral horn of spinal cord (Wright et al., 1997). We examined Er81 expression in lumbar DRG from E15 *myoNT3*/$NT3^{-/-}$ mice. As reported previously (Wright et al., 1997), parvalbumin expression was restored by NT3 expression in muscle (Figure 12D). Furthermore, we found that in these animals, numerous DRG neurons express Er81 (Figure 12H).

Finally, in order to define the neuronal cell type in which ErR81 exerts its role in controlling the development of central projections of proprioceptive afferents in the ventral spinal cord, we generated a targeted allele of *Er81* in which the first exon coding for the DNA binding ETS domain was flanked by *lox*P sites (Figure 16A). This mouse strain was crossed to $Isl1^{Cre}$ mice to eliminate Er81 expression exclusively in DRG neurons, motor neurons, and a subpopulation of dorsal interneurons in the spinal cord (Srinivas et al., 2001) but not in Er81 expressing interneurons in the intermediate and dorsal spinal cord.

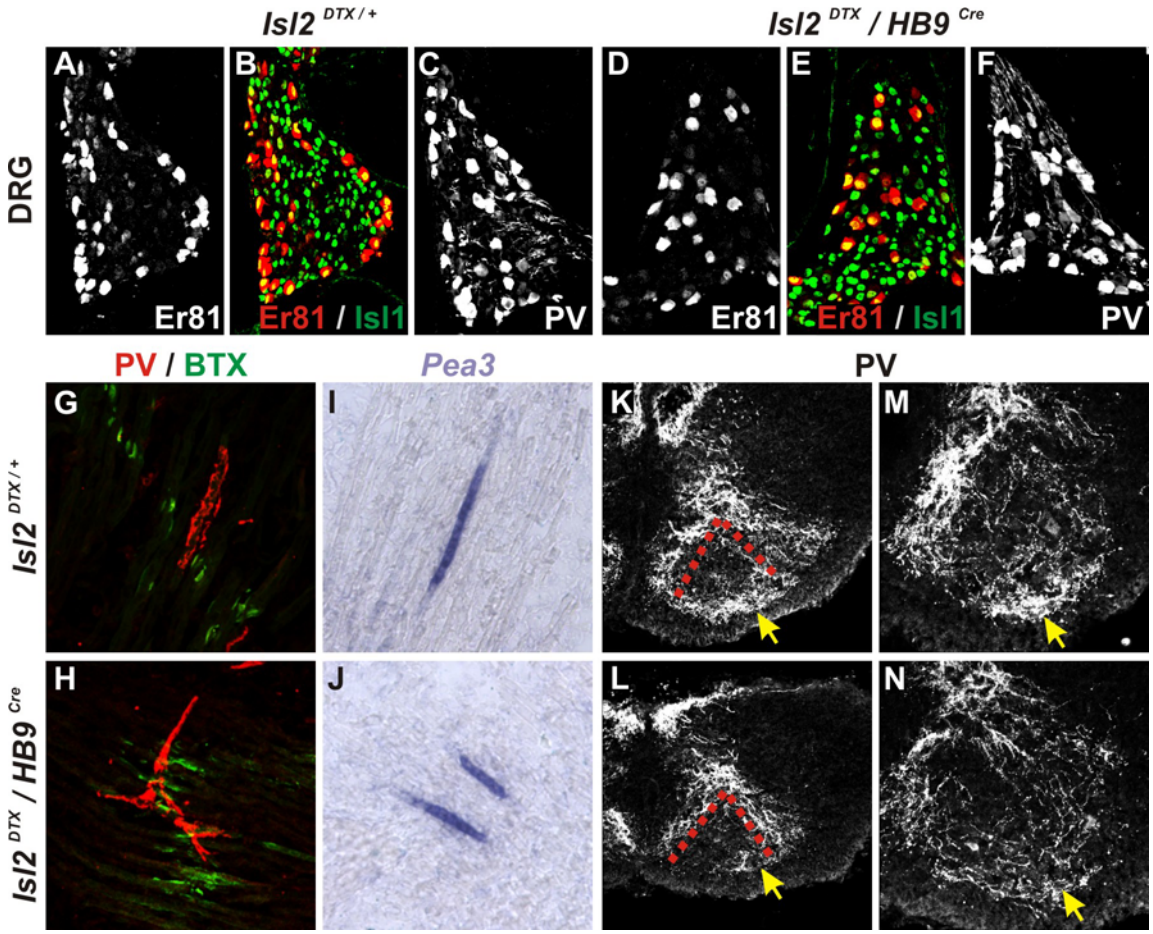


**Figure 15.** Proprioceptive afferent development in the absence of motor neurons

**(A–F)** Er81 (A, D), Er81 and Isl1 (B, E), and parvalbumin (C, F) immunocytochemistry on E17.5 brachial DRG from *Isl2*$^{DTX}$ (A–C) and *Isl2*$^{DTX}$/*Hb9*$^{Cre}$ (D–F) embryos. **(G–J)** Analysis of muscle spindle differentiation by parvalbumin (PV) and α-bungarotoxin (G, H) immunocytochemistry or *Pea3 in situ* hybridization (I, J) on forelimb muscles of E17.5 *Isl2*$^{DTX}$ (G, I) and *Isl2*$^{DTX}$/*Hb9*$^{Cre}$ (H, J) embryos. **(K–N)** Analysis of central projections of proprioceptive afferents by parvalbumin immunocytochemistry on spinal cords of E17.5 *Isl2*$^{DTX}$ (K, M) and *Isl2*$^{DTX}$/*Hb9*$^{Cre}$ (L, N) embryos. Arrows point to the ventral horn of the spinal cord where group Ia and II afferents normally terminate.

In *Isl1*$^{Cre}$/*Er81*$^{flox/-}$ mice, Er81 protein failed to be expressed in DRG neurons (Figure 16D, E), and parvalbumin positive proprioceptive afferents entered the spinal cord appropriately but terminated prematurely in the intermediate zone of the spinal cord (Figure 16G), similar to the findings in constitutive *Er81*$^{-/-}$ mice (Arber et al., 2000). These findings thus exclude a role for Er81 expressing interneurons in the intermediate zone of the spinal cord or Er81 expressing muscle spindles in affecting the targeting of proprioceptive afferents to the ventral spinal cord.

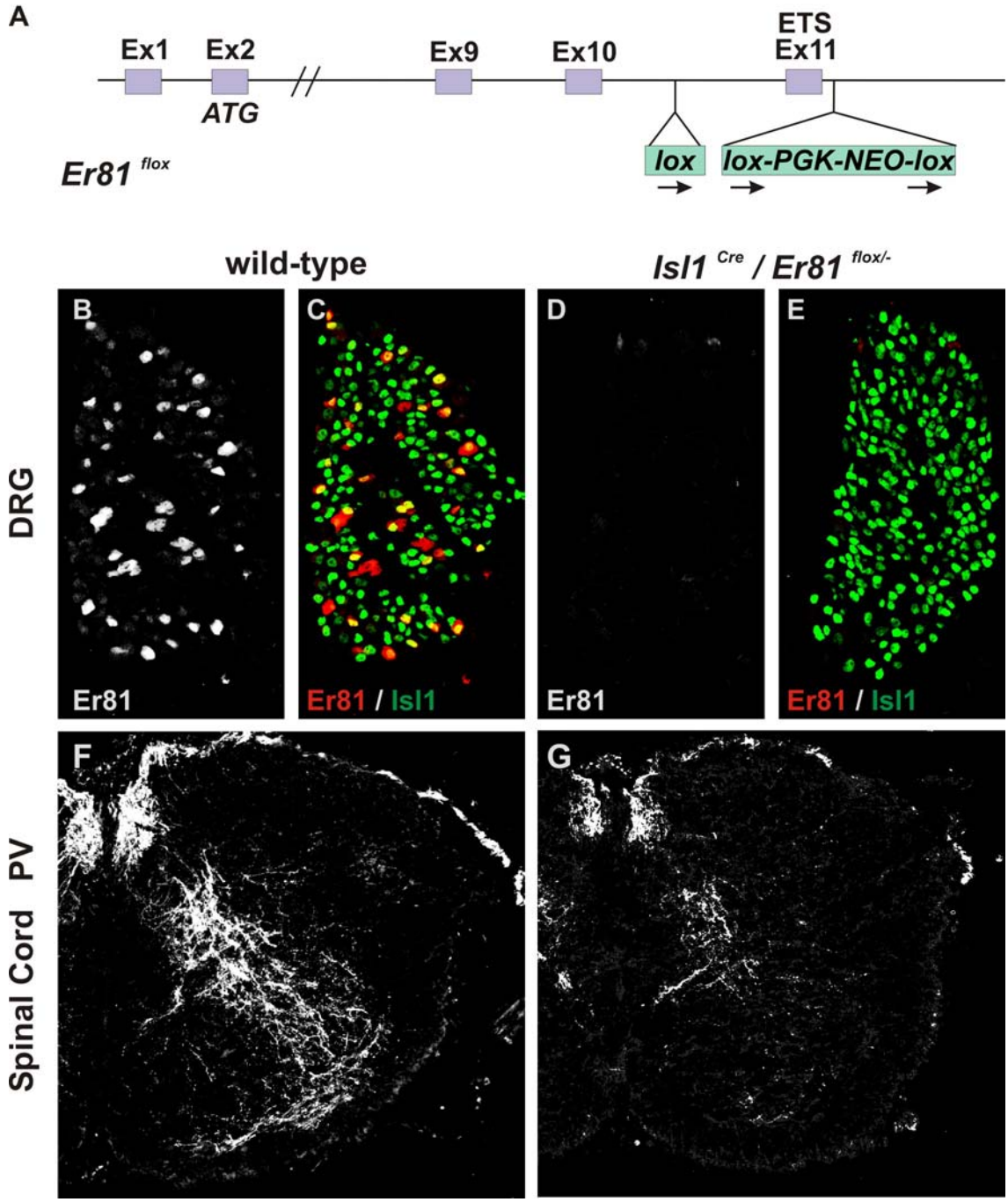


**Figure 16.** Er81 acts in proprioceptive afferents to control central connectivity

**(A)** Schematic diagram of targeting strategy for the *Er81*$^{flox}$ allele. **(B–E)** Er81 (B, D) and Er81/Isl1 (C, E) immunocytochemistry on E17.5 lumbar DRG from wild-type (B, C) and *Isl1*$^{Cre}$/*Er81*$^{flox/-}$ (D, E) embryos demonstrates absence of Er81 protein in DRG of *Isl1*$^{Cre}$/*Er81*$^{flox/-}$. **(F, G)** Analysis of central projections of proprioceptive afferents by parvalbumin immunocytochemistry on E17.5 lumbar spinal cords of wild-type (F) and *Isl1*$^{Cre}$/*Er81*$^{flox/-}$ (G) embryos.

Taken together, our findings suggest a model in which NT3 derived from the periphery is required to control expression of Er81 in proprioceptive DRG neurons. This ETS protein in turn controls, in a cell-intrinsic manner, the development of proprioceptive projections to the ventral spinal cord.

## DISCUSSION

NT3 is a powerful regulator of DRG neuronal number *in vivo* and proprioceptive neuron morphology *in vitro*, but relatively little is known about the effects of NT3 on proprioceptive axon extension and targeting during development *in vivo*. In this study, we have shown that in $Bax^{-/-}/NT3^{-/-}$ mice, proprioceptive DRG neurons survive through embryonic development. However, the surviving NT3 deprived neurons exhibit both peripheral and central projection defects. The peripheral processes of proprioceptive neurons fail to support muscle spindle differentiation and there is a reduction in the number of axons in the soleus nerve at P0. Central processes extend through the dorsal roots into the dorsal columns and branch and arborize in the intermediate spinal cord but fail to reach the motor neurons in the ventral horn. Furthermore, we find that in the absence of NT3 signaling, proprioceptive DRG neurons do not express the ETS family transcription factor Er81, and conversely NT3 is able to induce Er81 expression in E11 and E12 DRG neurons. Finally, we find that a peripheral source of NT3 is sufficient to induce Er81 expression by DRG neurons *in vivo*. These findings establish a role for peripheral NT3 in the regulation of proprioceptive afferent projections to motor neuron via the regulation of Er81 expression.

### NT3 REGULATES DRG NEURON NUMBER VIA CONTROL OF APOPTOSIS

Our findings demonstrate that elimination of Bax restores DRG neuronal number, as evidenced by a ~50% increase in the number of DRG neurons even in the absence of NT3 signaling. The increase in the number of DRG neurons in $Bax^{-/-}/NT3^{-/-}$ mice is comparable to that found in $Bax^{-/-}$ mice and is presumably due to the elimination of naturally occurring cell death.

There are two important aspects to NT3 regulation of DRG neuronal number. First, about 20% of DRG neurons, including the entire proprioceptive afferent population, express TrkC from E11 through postnatal life (Mu et al., 1993; Wright and Snider, 1995; White et al., 1996), and 20%–35% of DRG neurons are lost in TrkC mutant mice (Klein et al., 1994; Tessarollo et al., 1997). Therefore, NT3/TrkC signaling is thought to regulate survival of proprioceptive neurons in a manner analogous to regulation of the nociceptive population by NGF/TrkA signaling (Crowley et al., 1994; Smeyne et al., 1994). In contrast to findings in *TrkC*$^{-/-}$ mice, *NT3*$^{-/-}$ mice exhibit reductions of 60%–70% in DRG neuron number (Ernfors et al., 1994; Farinas et al., 1994; see also results from this study), raising the question as to the mechanism of action of NT3 on DRG neuron survival at early developmental stages.

The mechanism by which NT3 regulates DRG neuronal number remains unclear but several hypotheses have been put forward to explain the severe neuronal losses observed in the *NT3*$^{-/-}$ mice. Some studies have favored direct regulation of the cell cycle by NT3 (Verdi et al., 1996; Ockel et al., 1996) and therefore imply a control of neuron number through the regulation of precursor cell proliferation. Other studies have suggested that the proliferating precursors in the DRG that express TrkC undergo apoptosis in the absence of NT3, resulting in a depletion of neuronal precursors and in the generation of fewer neurons (ElShamy and Ernfors, 1996). Finally, still other studies of *TrkC*$^{-/-}$ and *NT3*$^{-/-}$ mice have suggested that failure of NT3 to activate TrkB during the proliferation stage of neurogenesis causes DRG precursors to exit the cell cycle prematurely, resulting in a smaller precursor pool (Farinas et al., 1996; Farinas et al., 1998). Characterization of Trk protein expression has demonstrated that sensory neuron precursors do not express TrkB or TrkC, suggesting that effects on the proliferating population are indirect (Farinas et al., 1998).

The current findings demonstrate that deletion of *Bax* restores DRG neuron number in the absence of NT3 signaling. Since the *Bax* deletion is thought selectively to affect the ability of DRG neurons or their precursors to enter an -apoptotic cell death program and does not influence their capacity to proliferate (Deckwerth et al., 1996; White et al.,

1998), our findings suggest that the effects of NT3 on DRG neuronal number are mediated via the inhibition of apoptosis. This conclusion leaves open the question of whether NT3 regulates the survival of precursors and/or postmitotic neurons. It is also possible that the early death of TrkB and TrkC neurons, or their precursors, could decrease the proliferation of neighboring cells. Our results do, however, argue against the idea that the direct regulation of proliferation by NT3 is likely to be a critical determinant of DRG neuronal number.

## NT3 REGULATES PERIPHERAL COMPONENTS OF THE PROPRIOCEPTIVE SYSTEM

Proprioceptive DRG neurons survive in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, but we find that their peripheral projections and associated muscle spindles are absent at P0. Thus, in the absence of NT3 signaling, there is a failure of peripheral proprioceptive axons either to grow toward their skeletal muscle targets during development, or alternatively a retraction of these processes with subsequent degeneration of the muscle spindles. Analysis of the expression of the zinc finger transcription factor Egr3 (Tourtellotte and Milbrandt, 1998) in muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$ at E15 and E17 revealed no staining for Egr3, arguing that Egr3 expression is never initiated in these mice. While we also never detected parvalbumin positive proprioceptive afferents in muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$, which may be caused by the low expression level of parvalbumin by these afferents, an independent tracing experiment revealed some peripheral axons in distal limb muscles with a morphology similar to annulospiral endings of group Ia or II afferents. An intriguing possibility would thus be that proprioceptive afferents are present transiently in muscle nerves of *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, but do not release neuregulin 1, which is thought to mediate initiation of muscle spindle differentiation (Hippenmeyer et al., 2002).

The lack of appropriate development of peripheral endings of proprioceptive afferents and associated sensory organs observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is analogous to our previous observations in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice in which TrkA+ nociceptive DRG neurons survive but their peripheral cutaneous projections are absent at P0 (Patel et al., 2000).

Together, these findings suggest a generality in the principle that neurotrophins are essential for the establishment and/or maintenance of peripheral sensory projections *in vivo*.

## NT3 REGULATES PROPRIOCEPTIVE AFFERENT PROJECTIONS INTO THE VENTRAL SPINAL CORD VIA REGULATION OF ER81 EXPRESSION

A striking finding of this study is that proprioceptive group Ia and II afferents fail to extend into the ventral spinal cord in the absence of NT3 signaling. Nevertheless, the initial extension of proprioceptive afferents into the dorsal roots and their initial arborization into the dorsal spinal cord proceed normally in the $Bax^{-/-}/NT3^{-/-}$ mutants. This finding is consistent with our observations in $Bax^{-/-}/NGF^{-/-}$ and $Bax^{-/-}/TrkA^{-/-}$ mice in which the extension of the central nociceptive projections into spinal cord proceeds normally in the absence of NGF/TrkA signaling (Patel et al., 2000). Since neither NT3 nor NGF are expressed in the dorsal roots or the dorsal horn of the spinal cord, the extension of the central sensory axonal processes appears not to require neurotrophin signaling.

Why, in $Bax^{-/-}/NT3^{-/-}$ mice, do the central group Ia and II projections extend only as far as the intermediate spinal cord and fail to project into the ventral horn? The targeting defect in $Bax^{-/-}/NT3^{-/-}$ mice is similar to that seen in mice lacking the ETS family transcription factor Er81. We find that proprioceptive DRG neurons in the $Bax^{-/-}/NT3^{-/-}$ do not express Er81 protein and that Er81 expression can be induced rapidly by application of NT3 in DRG explant cultures. NT3 expression by motor neurons does not appear to be required for the induction of Er81 in DRG. Moreover, peripheral NT3 can restore both the expression of Er81 in DRG as well as the development of central projections of proprioceptive afferents into the ventral spinal cord in $NT3^{-/-}$ mice. Since selective elimination of *Er81* in DRG and motor neurons recapitulated the $Er81^{-/-}$ phenotype, Er81 expression by interneurons in the intermediate zone of the spinal cord and by intrafusal muscle fibers is not crucial to proprioceptive-motor neuron targeting.

Together, these findings suggest that peripheral NT3 regulates central proprioceptive afferent projections through its ability to regulate Er81 in proprioceptive afferents.

Er81 is a member of the ETS family of transcription factors, an evolutionary conserved gene family characterized by sequence homology within the DNA binding ETS domain (reviewed in Bartel et al., 2000). It is unclear how Er81 regulates the targeting of proprioceptive afferents to motor neurons in the ventral horn of the spinal cord, but there is growing evidence that ETS family members play a role in regulating late steps in the establishment of sensory-motor neuron connectivity. In *Pea3*$^{-/-}$ mice, the axons of specific pools of motor neurons fail to invade and branch normally within their target muscles and the cell bodies of these motor neurons are mispositioned within the spinal cord (Livet et al., 2002). Limb derived signals, mediated in part by GDNF, regulate the expression of ETS proteins in motor neurons (Livet et al., 2002; Haase et al., 2002). Thus, an important implication of our findings, together with studies on Pea3, is that ETS genes act cooperatively at a late step of development to establish functional sensory-motor circuitry. In turn, ETS gene expression is regulated by neurotrophic factors released from peripheral target tissues.

It is interesting that NT3 regulates Er81 expression in proprioceptive sensory but not spinal motor neurons even though both of these neuronal classes express the NT3 receptor TrkC during embryonic development (Yan et al., 1993). This is one of several examples of differences in NT3 actions on these two types of neurons; for example, unlike proprioceptors, motor neurons do not require NT3 for survival. Little is known about differences in Trk intracellular signaling mediators that may be responsible for neuron class-specific effects.

One important question is the identity of downstream targets of Er81 that regulate the projection of proprioceptive axons. Given the specificity of the central proprioceptive targeting defect in *Er81*$^{-/-}$ and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, Er81 may be involved in regulating the expression of axon guidance and/or cell recognition molecules. Candidates include the type II cadherins. In developing chick DRG, type II cadherin family members are coexpressed with ETS family members Er81 and Pea3 in proprioceptive neurons and

spinal motor neurons. A matching of cadherin expression in proprioceptive sensory and motor neurons could therefore provide a basis for the selectivity with which sensory-motor neuron connections are formed (Price et al., 2002). It is possible that NT3 regulation of Er81 expression regulates the expression of type II cadherins in proprioceptive DRG neurons. Indeed, ectopic expression of Er81 results in the deregulation of at least one type II cadherin family member in the chick spinal cord (Price et al., 2002), and *Pea3*$^{-/-}$ mice show an altered profile of type II cadherin expression in motor neurons (Livet et al., 2002).

Finally, Er81 is unlikely to be the only transcription factor involved in controlling the development of central connectivity of group Ia and II afferents. Recently, a Runx family transcription factor Runx3 has been shown to be an essential regulator of proprioceptive DRG neuron development (Levanon et al., 2002; Inoue et al., 2002). *Runx3* mutant mice exhibit severe limb ataxia due to disruption of monosynaptic connectivity between proprioceptive afferents and motor neurons. The central projection defect in the *Runx3*$^{-/-}$ mice is more severe than that reported in *Er81*$^{-/-}$ mice (Arber et al., 2000) and than that reported here in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Specifically, in *Runx3*$^{-/-}$ mice, proprioceptive DRG neurons fail to extend central processes into the intermediate spinal cord. In contrast, in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Er81*$^{-/-}$ mice, proprioceptive afferents extend as far as the intermediate zone but fail to extend into the ventral horn. These findings raise the possibility that Runx3 and Er81 function coordinately for proper targeting of group Ia and II afferents to the ventral horn.

## ROLES OF NEUROTROPHIC FACTORS IN AXON TARGETING

Our study and the prior studies of Haase et al. (2002) and Ma et al. (2002) establish an important role for neurotrophic molecules in regulating axon targeting. Surprisingly, their effect on targeting is not due to the intensively studied chemotropic function of these molecules (see O'Connor and Tessier-Lavigne, 1999; Tucker et al., 2001, and references therein). In the circuit considered here, NT3 is expressed by motor neurons well prior to projection of proprioceptive afferents into the ventral horn (see Wright et al., 1997, and references therein). We show here that proprioceptive afferents project toward the ventral

horn even if motor neurons are ablated genetically. In addition, it has been shown that a central source of NT3 is unimportant in the central targeting of proprioceptive axons, since central NT3 can be neutralized by anti-NT3 antibodies without affecting the projections of proprioceptive afferents (Oakley et al., 1995). Our evidence demonstrates that NT3 regulates targeting of proprioceptive afferents to motor neurons via a different mechanism - the regulation of Er81 expression and presumed subsequent effects on gene transcription through a peripheral source of NT3. At a later stage, local regulation of proprioceptive axon branching in the vicinity of the motor neuron pools may involve Wnt signals derived from motor neurons (Krylova et al., 2002).

Since there is a peripheral projection defect in $Bax^{-/-}/NT3^{-/-}$ mice, it could also be argued that target-derived factors in addition to NT3 are required to direct proprioceptive afferents to the ventral horn. Two lines of evidence suggest that NT3 has the predominant role. In chick, when proprioceptive axons are deprived of their peripheral targets by limb bud ablation, exogenous application of NT3 is sufficient to direct projection of muscle afferents appropriately within the spinal cord (Oakley et al., 1997). Furthermore, peripherally supplied NT3 is sufficient to direct central projections to motor neuron pools even if the peripheral proprioceptive axon is misrouted in the periphery and therefore exposed to atypical peripheral influences (Oakley and Karpinski, 2002). Interestingly, target-derived NT3 also exerts important influences on proprioceptive afferent-motor neuron connections at later developmental stages and into maturity. Thus, intramuscular injections of NT3 rescue the functional deficit in group Ia and II synaptic transmission observed in $Egr3^{-/-}$ mice where proprioceptors lose their peripheral target end organ due to progressive degeneration of muscle spindles after birth (Tourtellotte and Milbrandt 1998; Chen et al., 2002). Furthermore, administration of NT3 rescues the defect in group Ia and II synaptic transmission that results from separation of proprioceptive afferents from their targets by axotomy in fully mature animals (Mendell et al., 1999). Thus, a single target-derived neurotrophic factor appears to regulate both the development and function of this sensory-motor circuit.

# METHODS

## ANIMALS

*Bax*$^{-/-}$ mice on a 129/Bl6 background (from Dr. Stan Korsemeyer) were crossed with *NT3*$^{+/-}$ mice also on a 129/Bl6 background to generate *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. *Bax*$^{-/-}$ and *NT3*$^{-/-}$ mice maintained on a pure C57Bl/6 genetic background (Jackson Labs) were also crossed to produce *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Results from the two genetic backgrounds were indistinguishable. Genotypes were confirmed by tail DNA PCR (Deckwerth et al., 1996; Wright et al., 1997). *Er81*$^{flox}$ mice were generated by the integration of *lox*P sites 5′ and 3′ of exon 11 in the *Er81* locus in a targeting strategy analogous to Arber et al. (2000). *Isl1*$^{Cre}$, *Hb9*$^{Cre}$, and *Isl2*$^{DTX}$ mice have been described previously (Srinivas et al., 2001; Yang et al., 2001; Pun et al., 2002). The generation of *myoNT3* mice was described previously (Wright et al., 1997).

## NEURON AND NERVE FIBER COUNTS

Neuron and axon counts in the different mutants were conducted as described previously in detail in Patel et al. (2000).

To measure the cross-sectional area of parvalbumin positive DRG neurons at E15 and P0, images of lumber DRG sections were captured at 20x magnification and analyzed using the NIH image software. A minimum of 15 lumbar DRG sections from each mouse was captured. The outline of parvalbumin positive cell bodies were manually traced and diameter and cross-sectional area measurements were recorded.

## IMMUNOHISTOCHEMISTRY

E15, E17, and P0 mice were intracardially perfused with 4% paraformaldehyde (PFA). Tissue was dissected, postfixed in 4% PFA, washed extensively in PBS, and incubated in 30% sucrose/PBS. DRG, spinal cord, and limb sections were cut on a cryostat at 10 to 20μm. Sections were incubated in primary antibody overnight. The following antibodies

were used: goat anti-parvalbumin (1:500, Swant, Switzerland), guinea pig anti-Isl1 (Arber et al., 2000), rabbit anti-parvalbumin (1:5000, Swant, Switzerland), rabbit anti-Egr3 (1:500, Santa Cruz, Santa Cruz, CA), and rabbit anti-Er81 (1:12,500; Arber et al., 2000). Sections were washed in PBS and incubated with the appropriate Cy-2- or Cy-3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), Alexa-488-conjugated secondary antibodies (Molecular Probes), or fluorescently labeled bungarotoxin (Molecular Probes) and prepared for visualization. To confirm the absence of Egr3 immunoreactivity in E15 and E17 $Bax^{-/-}/NT3^{-/-}$ muscles, we examined more than 100 hindlimb and forelimb sections at E15 and more than 200 sections at E17 from a total of three animals at each age.

## DiI LABELING

To label the central DRG axon projections, DiI crystals (Molecular Probes) were placed directly in the DRG at E15, E17, and P0. The tissue was incubated at 37°C in 4% paraformaldehyde and monitored periodically to assess the extent of labeling. Spinal cords were sectioned at 75 μm on a vibratome for visualization. For labeling of peripheral axon projections, DiI crystals were placed in the sciatic nerve proximal to the tibial and common peroneal branch points of E17 embryos and processed as above.

To quantify DiI labeled afferents, mean intensity measurements in defined regions of the intermediate zone and ventral horn of spinal cord were obtained using the NIH Image analysis software. Photoshop images were first converted to gray scale and inverted. Images were then imported into NIH Image, and mean intensity measurements of DiI-labeled afferents in a given area were recorded. Background corrections were made for each section. Measurements were made in a fixed area at a defined point for all sections examined. The data were expressed as the ratio of mean density measurements in the $Bax^{-/-}/NT3^{-/-}$ mice versus the $Bax^{+/+}/NT3^{+/+}$ mice.

## *IN SITU* HYBRIDIZATION

*In situ* hybridization was performed on fixed tissue sections according to previously described protocols (Wright and Snider 1995; Patel et al., 2000) using digoxigenin-labeled sense and antisense riboprobes for *Er81* and *Pea3*.

## EXPLANT CULTURES

For Er81 induction studies, E11.5 and E12.5 wild-type DRG were dissected and whole explants cultured in DMEM/F12 medium supplemented with 10% FCS and 2 mM L-glutamine. Cultures were maintained under conditions of no neurotrophin, 10 ng/ml NT3, or 50 ng/ml NGF up to 18 hours. At different time intervals, explants were washed once for 5 min in PBS, fixed for 20 min on ice in 4% PFA, washed again in PBS, incubated in 30% sucrose/PBS overnight, and embedded the next morning using the Tissue-Tek OCT compound. After freezing, 9-μm thin sections were cut and antibody staining performed according to standard procedures.

*Bax*$^{-/-}$ DRG explant cultures were performed as described in Patel et al. (2000).

# CHAPTER 4

# GENETIC TRACING OF NEURONAL SUBPOPULATIONS

# INTRODUCTION

Any attempt to study neuronal circuit formation, maturation or maintenance *in vivo* will ultimately rely on the ability to visualize and trace axons and dendrites of the individual neuronal components constituting the specific neuronal circuit of interest. Therefore, it is necessary to develop tools allowing selective labeling of specific neuronal subpopulations by expression of distinct cytoplasmic or membrane-linked markers, such as β-galactosidase or fluorescent molecules like enhanced green fluorescent protein (EGFP), thereby rendering neurons distinguishable from the surrounding neuropil allowing them to be identified and observed *in vivo*. One way to achieve this is by transfection of neurons with viral constructs driving expression of specific marker or reporter genes (Washbourne and McAllister, 2002), which can either be done directly *in vivo* in the living animal or in slices of cultured nervous tissue. Another frequently used method is to directly introduce a dye or tracer molecule into the living animal in the specific area of interest, which will then selectively label neurons and/or corresponding neuronal tracts through retrograde or anterograde transport (Gan et al., 1999; 2000). Despite the fact that all these techniques have been widely and successfully employed in the past, non-invasive techniques for labeling neuronal subpopulations in living animals, for example, by generation of transgenic animals, would be of great advantage. Likewise, side effects due to expression of the tracer itself could be minimized, whereas reproducibility with respect to the identity of labeled neurons would be maximized, as the molecular tracer would be stably inherited to the next generation.

In order to label different neuronal subpopulations in the mammalian nervous system we turned to a classic transgenic strategy based on pronuclear injections aimed at generating novel mouse tracer lines expressing subcellularly tagged variants of EGFP and nuclear-targeted β-galactosidase from a bi-cistronic message. In addition, we were able to further limit the number of neurons expressing the molecular tracers by combining conventional transgene expression with the Cre-*lox*P-system widely used for conditional gene expression (Nagy, 2000; Lewandoski, 2001), adding a second component of spatio-temporal control to transgene expression.

We decided to use the well-established modified *Thy1* gene regulatory region as a neuron-specific regulatory element driving neuronal transgene expression at high levels throughout the nervous system.

The *Thy1* gene encodes a GPI-linked cell-surface glycoprotein of the immunoglobulin superfamily expressed by thymocytes (hence its name: *thymus cell antigen 1*, *theta*) and other nonneuronal cell types as well as by projections neurons in many areas of the central and peripheral nervous system (Morris, 1985; 1992; Barlow and Huntley, 2000). The *Thy1* gene regulatory region has been used previously to drive high-level transgene expression specifically in the nervous system (Gordon et al., 1987; Kelley et al., 1994; Aigner et al., 1995; Caroni, 1997). To this end a *Thy1* expression cassette was constructed removing the entire coding region of the murine *Thy1* gene, at the same deleting regulatory sequence elements mostly present within a particular intron responsible for *Thy1* expression in nonneuronal tissues such as the thymus or connective tissue (Vidal et al., 1990; Aigner et al., 1995; Caroni, 1997). Analysis of several transgenic mouse lines generated by pronuclear injection of different *Thy1*-derived transgene cassettes revealed strong neuronal transgene expression throughout the nervous system as well as a high variability amongst individual transgenic mouse lines with respect to the number and identity of neuronal subpopulations expressing the transgene. Transgene expression driven by the modified *Thy1* regulatory elements therefore appears to be highly sensitive to the chromosomal integration site making it possible to obtain several different transgenic mouse lines by injecting an identical transgene construct.

To visualize not only different subpopulations of neurons but also distinct subcellular compartments within those neurons we generated EGFP encoding fusion constructs allowing either membrane targeting or presynaptic localization of EGFP. Membrane targeting was achieved by fusing a modified membrane localization domain present in the myristoylated alanine rich protein kinase C substrate (MARCKS) to the amino terminus (N-terminus) of EGFP (Aderem, 1992; Wiederkehr et al., 1997). Presynaptic localization was obtained by fusion of the synaptic vesicle protein synaptophysin (Syp) to the N-terminus of EGFP (De Paola et al., 2003). Furthermore, by using an internal ribosome entry sequence (IRES) coupled to a *nls-lacZ* reporter gene encoding nuclear-targeted β-galactosidase, we were able to generate transgenic mice expressing subcellular-targeted

EGFP variants as well as nuclear-targeted β-galactosidase from a single bi-cistronic message, thereby greatly facilitating initial screening and mapping of individual transgenic lines. Moreover, by introducing a transcriptional stop sequence (SV40 polyadenylation signal) at the 5' end of the transgene cassettes flanked by *lox*P sites (*lox*P-*stop*-*lox*P, *LSL* cassette) we could further limit and define the number of neurons expressing the transgene by linking transgene expression directly to expression of Cre recombinase in a conditional *Cre*-*lox*P based manner.

Using the outlined genetic strategy we were able to establish several novel EGFP tracer mouse lines, which were analyzed with respect to transgene expression in the spinal cord and in DRG sensory neurons.

# RESULTS AND DISCUSSION

## MOLECULAR TAGGING OF NEURONAL SUBPOPULATIONS

Expression of nuclear-targeted β-galactosidase in newly generated *Thy1* lines allowed fast and easy mapping of transgene expression in different transgenic lines by comparing X-gal staining patterns to expression patterns observed in a $\beta$-$Actin^{LSL\text{-}nlslacZ}$ reporter mouse line, which expresses nuclear-targeted β-galactosidase following activation by Cre recombinase (see Figure 17A, B), but in contrast to *Thy1* transgenic lines all Cre expressing cells are labeled in the reporter line (compare Figure 17A, B with 17C, D). As previously reported (Caroni, 1997; Feng et al., 2000) individual transgenic mouse lines displayed strong differences in the number and identity of labeled neurons as well as in transgene expression levels. Out of nine originally obtained $Thy1^{LSL\text{-}MGFP\text{-}IRES\text{-}nlslacZ}$ ($Thy1^{mGFP\text{-}INLA}$) transgenic mouse lines a total of six lines showed transgene expression (67%) after crossing with $Isl1^{Cre}$ mice driving expression of Cre recombinase in spinal motor neurons and some dorsal interneurons as well as in DRG sensory neurons. Four out of these six lines, however, showed either weak transgene expression or expressed only in very few neurons ("$Thy1^{mGFP\text{-}INLA}$ (Few)", Figure 17C, E) and therefore were not analyzed further, while the two other transgenic lines showed strong transgene expression in many neurons ("$Thy1^{mGFP\text{-}INLA}$ (Many)", Figure 17D, F, G).

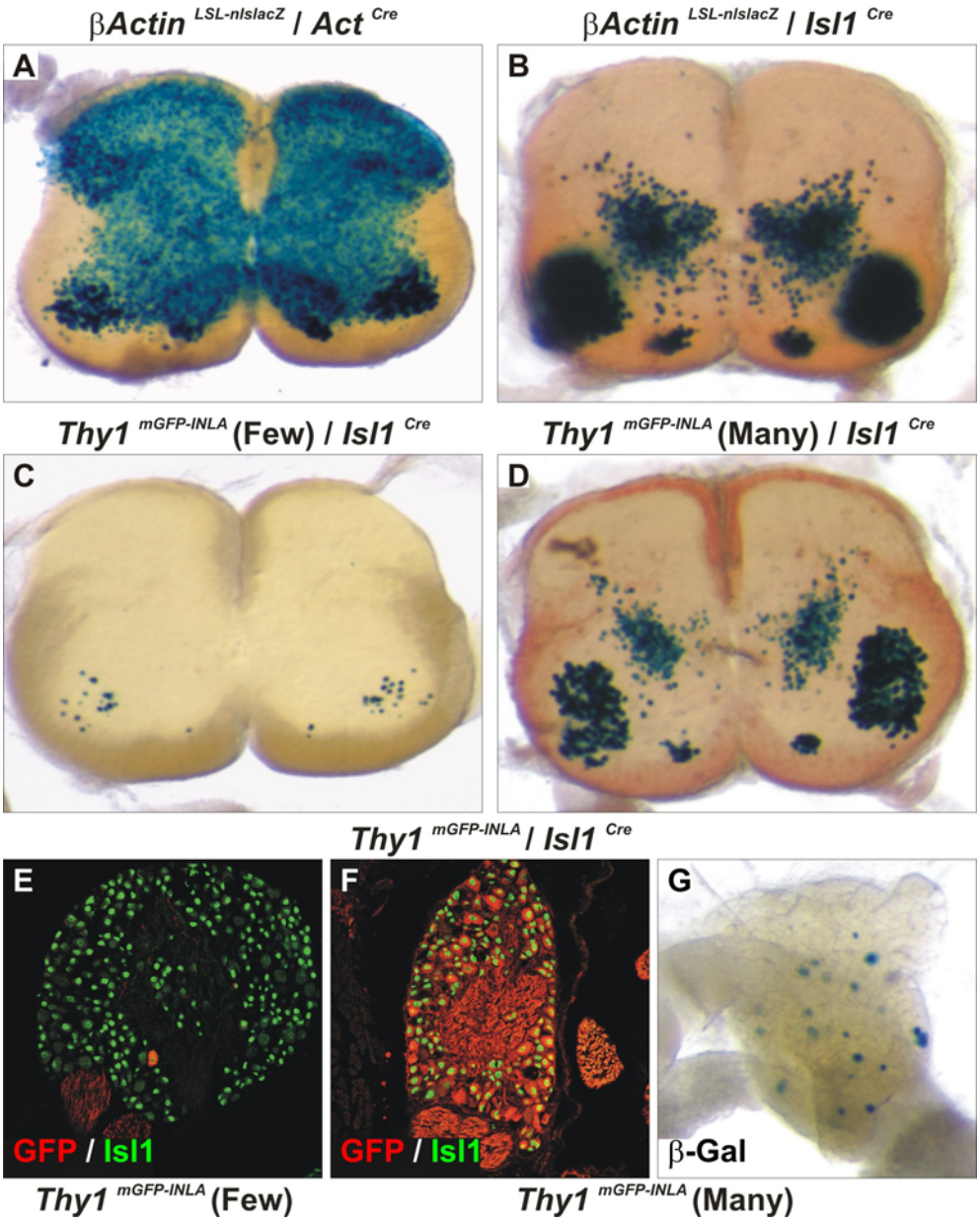


**Figure 17.** Conditional activation of *Thy1* transgene expression in spinal cord and DRG

Analysis of *Thy1* directed transgene expression of *MGFP-IRES-nlslacZ* (*mGFP-INLA*) after activation by *Isl1*$^{Cre}$ in slices of spinal cord (C, D) or DRG (E-G). **(A-D)** X-gal staining in slices of spinal cord from *β-Actin*$^{LSL\text{-}nlslacZ}$ reporter mice (A, B) or different *Thy1* transgenic mouse lines (C, D). Nuclear-targeted β-galactosidase expression in reporter mice labels all spinal cells when activated by *Actin*$^{Cre}$ (A) or all Isl1 positive neurons when activated by *Isl1*$^{Cre}$ (B). In contrast, different *Thy1* transgenic mice express nuclear-targeted β-galactosidase in distinct neuronal subsets of Isl1 positive neurons. In some lines only few motor neurons of the MMC and LMC are labeled, whereas in others many motor neurons as well as interneurons in the intermediate region of the spinal cord express the transgene after activation by *Isl1*$^{Cre}$. **(E, F)** Double labeling experiment to detect GFP and Isl1 in *Thy1* transgenic lines with only few DRG neurons (E) or many DRG neurons labeled (F). **(G)** X-gal staining of DRG whole mount.

The remaining two lines, SA-38 and SA-39, differed mainly in the number and identity of transgene expressing neurons after activation with *Isl1*$^{Cre}$, while transgene expression levels were similar (Figure 18A-F; Figure 19A, E). Both lines displayed comparable patterns of transgene expression in spinal motor neurons and spinal interneurons, although generally slightly more spinal neurons expressed the transgene in line SA-39 compared to line SA-38 (compare Figure 18A, B with 18D, E). In addition, neuronal expression analyzed after activation by *Actin*$^{Cre}$ also revealed in both mouse lines comparable patterns of transgene expression, such as in layers of the cerebral cortex, in the hippocampus and the amygdala (data not shown). Moreover, line SA-39 displayed transgene expression in granule cells of the cerebellum, which was not observed in transgenic mice of line SA-38 (data not shown).

Combining the *Cre-lox*P system with a conventional transgenic approach as described here provides a powerful tool for limiting the number of neurons expressing the transgene to a defined neuronal subpopulation. This becomes readily clear when comparing the dramatically different expression patterns obtained after activation of transgene expression by introducing either the *Isl1*$^{Cre}$ or the *Lim3*$^{Cre}$ allele, respectively (compare Figures 18D, E with 18G).

Transgenic *Thy1*$^{LSL\text{-}SypGFP\text{-}IRES\text{-}nlslacZ}$ (*Thy1*$^{SypGFP\text{-}INLA}$) mice displayed qualitatively similar expression profiles compared to those observed in *Thy1*$^{mGFP\text{-}INLA}$ mice (Figure 19). Out of eleven initially obtained lines eight lines expressed the transgene (73%) after activation by *Isl1*$^{Cre}$. Four of these showed strong levels of transgene expression in DRG sensory neurons, including proprioceptive sensory neurons, since muscles spindles as well as GTOs, the peripheral sensory organs innervated by Ia and Ib proprioceptive afferents, respectively, were labeled by EGFP expression (line SA-40; data not shown). Moreover, also some spinal interneurons as well as motor neurons, as assessed by EGPF expression at the NMJ in the *gluteus maximus* muscle, expressed the transgene after activation with *Isl1*$^{Cre}$ (data not shown). Moreover, in one line (line SA-41) a single synaptic targeting layer within the dorsal horn of the spinal cord was labeled by SypGFP expression at synaptic terminals (Figure 19B, C).

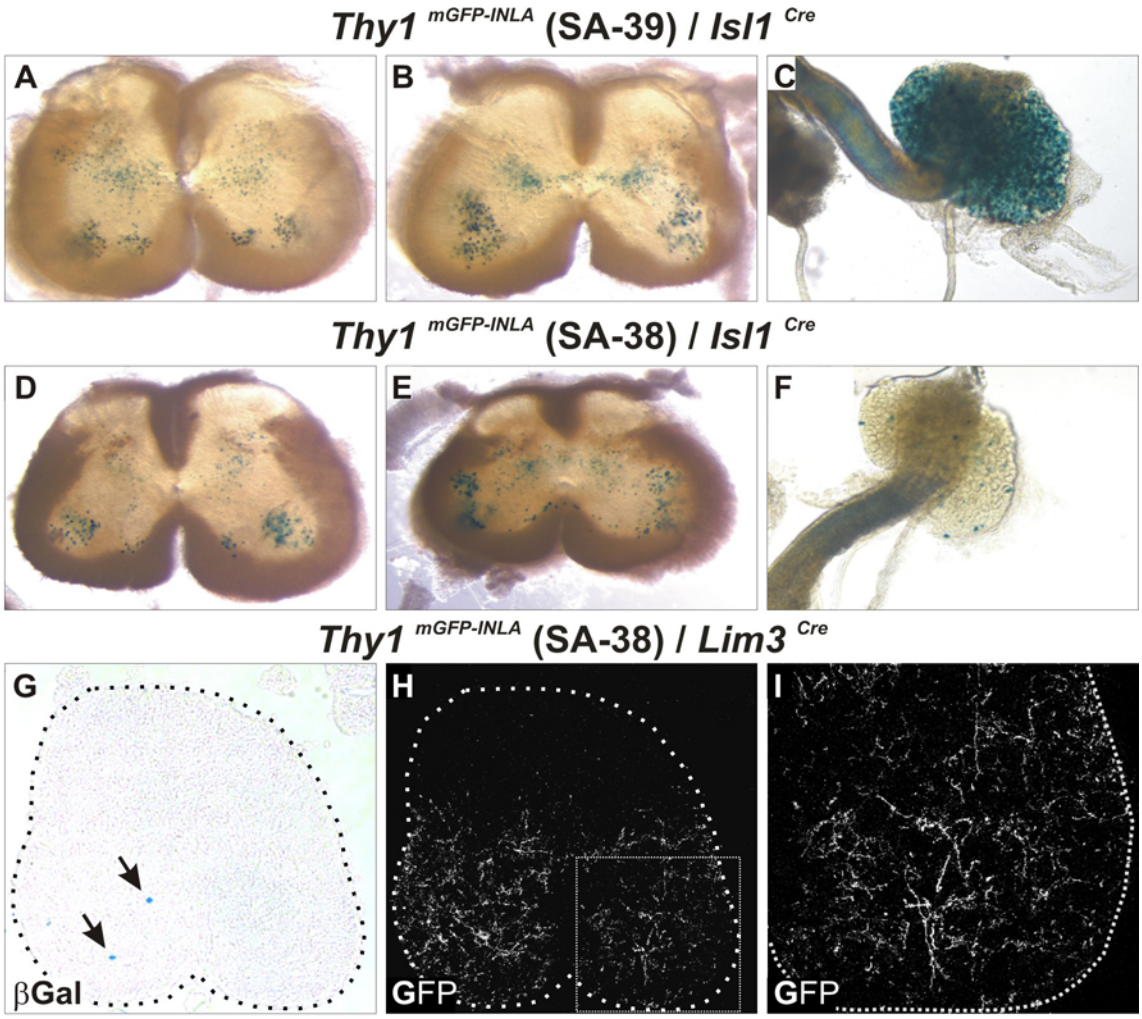


**Figure 18.** Selective labeling of distinct neuronal subpopulations in spinal cord and DRG

*Thy1* directed transgene expression of *MGFP-IRES-nlslacZ* (*mGFP-INLA*) after activation by *Isl1*$^{Cre}$ (A-F) or *Lim3*$^{Cre}$ (G-I) visualized by X-gal staining (A-G) or by immunohistochemical analysis of GFP expression on thin sections of lumbar spinal cord (H, I). **(A, B)** Line SA-39/*Isl1*$^{Cre}$ mice express nuclear-targeted β-galactosidase in both MMC and LMC motor neurons as well as in some interneurons in the intermediate region of the spinal cord. **(C)** X-gal straining labels basically all sensory neurons in DRG whole mounts of line SA-39/*Isl1*$^{Cre}$. **(D, E)** Line SA-38/*Isl1*$^{Cre}$ show similar spinal expression patterns compared to line SA-39/*Isl1*$^{Cre}$ (A, B), but generally slightly less spinal neurons are labeled. **(F)** Only some sensory neurons express the transgene in DRG whole mounts of line SA-38/*Isl1*$^{Cre}$. **(G)** Activation of transgene expression in line SA-38 by *Lim3*$^{Cre}$ labels only very few spinal interneurons by nuclear-targeted β-galactosidase on thin sections of lumbar spinal cord (arrows). **(H, I)** Consecutive section to image in (G) taken at 22x magnification (H). Dotted box in (H) represents image shown in (I) at higher magnification of 50x.

In summary, the combination of conditional activation of transgenic reporters based on EGFP variants and nuclear-targeted β-galactosidase by Cre recombinase represents a suitable approach for tracing specific subpopulations of neurons *in vivo*.

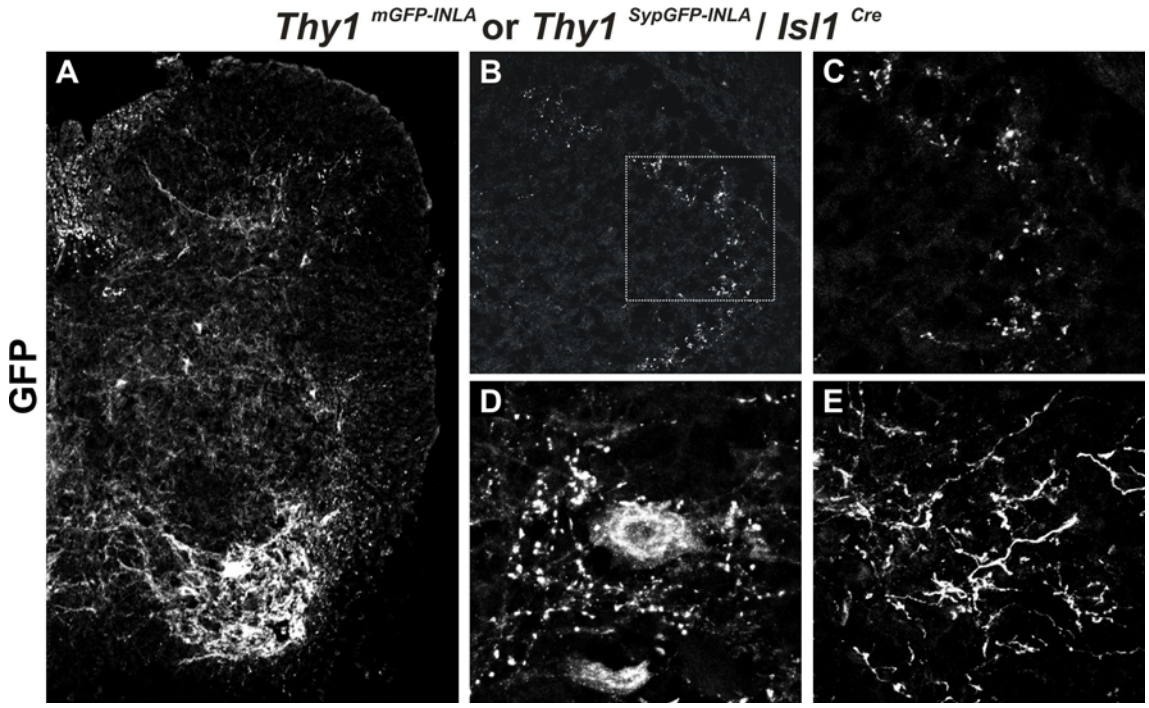


**Figure 19.** Molecular tagging of neuronal subpopulations in mouse spinal cord

*Thy1* directed transgene expression of *MGFP-IRES-nlslacZ* (*mGFP-INLA*; A, E) or of *SypGFP-IRES-nlslacZ* (*SypGFP-INLA*; B-D) following activation with *Isl1*$^{Cre}$ visualized by immunohistochemical analysis of EGFP expression on sections of spinal cord (A-E). **(A)** Spinal cord hemisection of a line SA-38/*Isl1*$^{Cre}$ mouse showing MGFP labeling in a subset of motor neurons and interneurons. **(B)** A single synaptic targeting layer in the dorsal horn is labeled by SypGFP in spinal cord of line SA-41/ *Isl1*$^{Cre}$ mice. **(C)** Higher magnification of the inset shown in (B) as a dotted box to visualize individual synapses. **(D)** High magnification of part of the ventral horn showing strong SypGFP labeling in the vicinity of a motor neuron cell body in the spinal cord of a line SA-40/*Isl1*$^{Cre}$ mouse. **(E)** Single MGFP labeled axons can be traced in the dorsal horn of spinal cord in line SA-39/ *Isl1*$^{Cre}$ mice.

Moreover, Cre recombinase-mediated excision of transcriptional stop sequences in *Thy1*-derived transgenes appears to occur with good efficacy, as about 67% and 73% of the original transgenic lines showed positive transgene expression after Cre recombinase-mediated activation, respectively. This percentage is comparable to the previously reported one of 84% for conventional *Thy1*-derived transgenes (Feng et al., 2000).

In addition, we also generated several interesting new mouse lines with highly specific expression patterns in cases where transgene expression was not conditionally regulated by a Cre-*lox*P based strategy (De Paola et al., 2003; Hippenmeyer et al., 2005).

One mouse line (SA-36) was found to selectively express membrane-targeted EGFP in a subpopulation of non-peptidergic DRG and trigeminal ganglion sensory neurons binding the isolectin B4 (IB4) from *Griffonia simplicifolia* (Figure 20A-C). These DRG neurons have been shown to be cutaneous sensory neurons undergoing a developmentally regulated switch in neurotrophic factor receptor profile reflected by upregulation of Ret expression concomitant with a downregulation of TrkA expression during the first three weeks of postnatal life (Figure 20C-E, Molliver et al., 1997). Centrally they innervate specific targets in the so-called substania gelantinosa or lamina IIi according to the classification of Rexed of the spinal cord (Snider and McMahon, 1998), which is also reflected by the strong band of EGFP expression in this area seen in transgenic mice of this line (Figure 20H). Moreover, EGFP expression did not co localize with CGRP, substance P/tachykinin 1 (Tac1), galanin or neuropeptide Y (Npy) expression (Figure 20G; data not shown), further confirming the identity of the transgene expressing DRG neurons as being non-peptidergic cutaneous neurons binding to IB4.

Endogenous *Thy1* expression steadily increases from early postnatal life onwards and is maintained throughout adult life. Similarly, *Thy1*-directed transgene expression usually begins around P4 and is maintained at high levels in the mature nervous system. However, in some transgenic lines transgene expression was already detectable as early as embryonic day 13.5 (E13.5), most likely reflecting epigenetic effects due to the specific chromosomal integration site (Feng et al., 2000; Hippenmeyer et al., 2005; data not shown).

In conclusion, like previously reported, we detected large variations between different transgenic lines with respect to the identity and number of neurons and the strength of transgene expression. However, there was no correlation between the number or identity of neurons and the level of transgene expression, the latter likely reflecting differences in transgene copy number, which can range from 1 to over 50 copies arranged in tandem as concatemers. Generally, irrespective of the type of EGFP fusion proteins expressed some lines showed strong transgene expression in many neurons whereas others displayed highly restricted expression patterns in only very few neurons of a particular subtype.

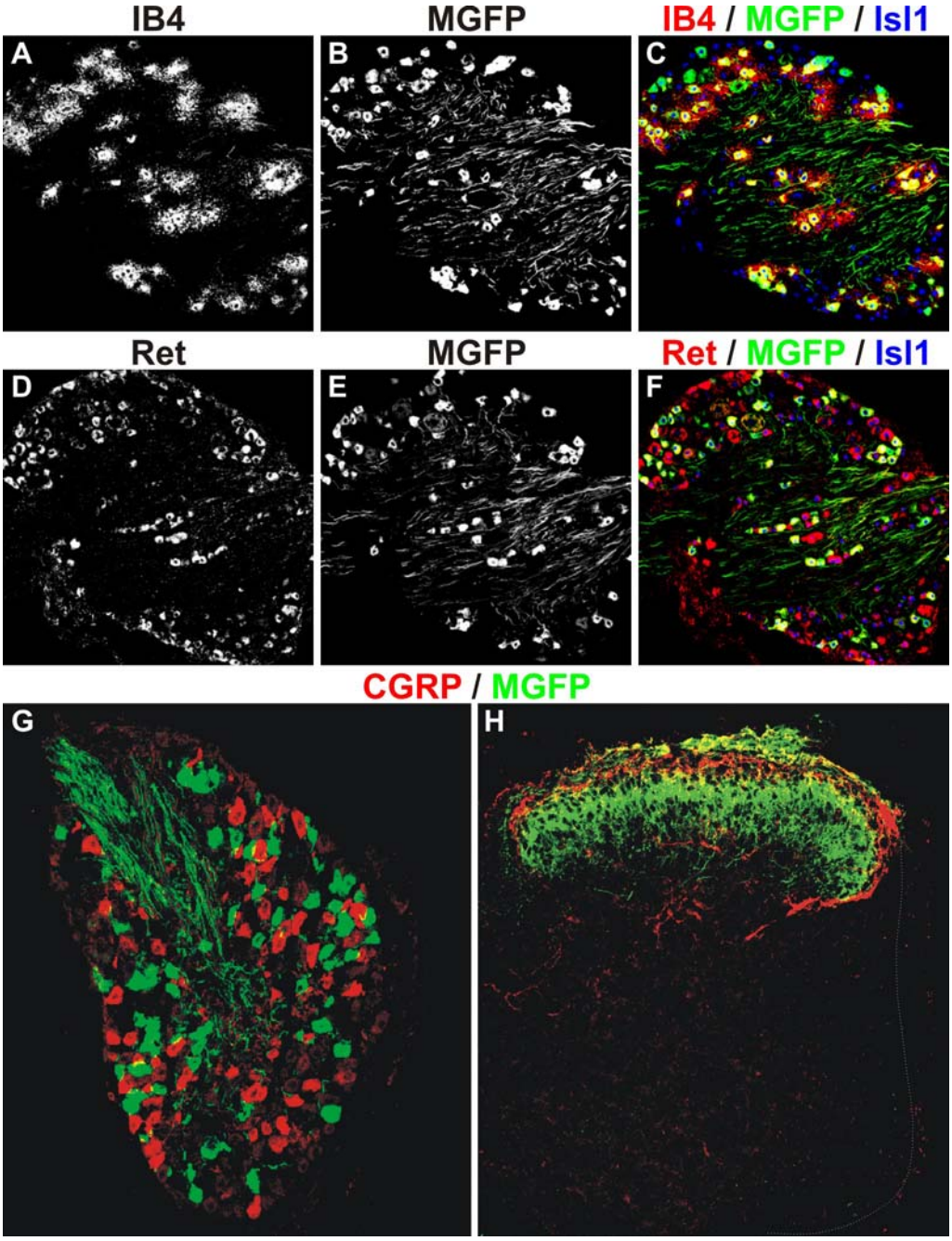


**Figure 20.** MGFP expression in IB4-binding DRG sensory neurons

*Thy1* directed transgene expression of *MGFP-IRES-nlslacZ* in line SA-36 visualized by immunohistochemical analysis of MGFP in adult lumbar DRG (A-G) and spinal cord (H).
**(A-C)** Triple labeling experiment to compare IB4-binding (A, C: red) with MGFP expression (B, C: green) in Isl1 positive DRG sensory neurons (C: blue). Note colocalization of IB4-binding and MGFP expression in adult DRG neurons (C). **(D-F)** Triple labeling experiment to compare Ret (D, F: red) and MGF expression (E, F: green) in Isl1 positive DRG sensory neurons (F: blue). MGFP expressing DRG neurons represent a part of the Ret positive subpopulation of cutaneous DRG neurons (F). **(G, H)** Double labeling experiment to detect CGRP (red) and MGFP expression (green) in adult lumbar DRG neurons (G) and spinal cord (H) showing lack of coexpression of CGRP and MGFP.

Most likely, these differences in transgene expression profiles are a result of differences in chromosomal integration sites, which vary from line to line. In contrast to the pronounced line-to-line variations observed, there was basically no variation detectable between different animals of the same transgenic mouse line, demonstrating stable genetic inheritance of expression patterns over several generations. Thus, with the genetic strategy outlined in this section we were able to establish several novel genetic tracer mouse lines allowing detailed anatomical analysis of specific neuronal subclasses by distinct expression of EGFP targeted specifically to membranes or presynaptic terminals.

# METHODS

## ANIMALS

The original 6.5 kb murine *Thy1* backbone vector as well as a MARCKS-EGFP fusion construct (see below) were generously provided by Vincenzo De Paola and Pico Caroni (Friedrich Miescher Institute for Biomedical Research, Basel). To facilitate transgene insertion into the *Thy1* backbone vector as well as subsequent purification for pronuclear injections we subcloned a 5.5 kb *Eco*RI – *Kpn*I fragment into a modified pGEM-7 vector (Promega), thereby flanking the entire *Thy1* backbone at the 5' and 3'end by two *Pme*I sites while at the same time deleting one kb from the 3' end of the original *Thy1* vector. To facilitate insertion of transgene cassettes additional unique cloning sites (*Asc*I and *Mlu*I) were introduced into the previously present unique *Xho*I site of the modified *Thy1* backbone.

Membrane targeting of EGFP was achieved by fusion of the first 41 amino acids of human MARCKS to the N-terminus of EGFP (Clontech). To enhance membrane localization an N-terminal alanine to cysteine (Ala→Cys) mutation in position +3 of MARCKS had been introduced, thereby creating a palmitoylation site. Presynaptic targeting of EGFP was obtained by N-terminal fusion of full-length synaptophysin to EGFP.

Transgenic mice were generated by pronuclear injection of fertilized oocytes with gel-purified DNA using standard techniques (Hogan et al., 1994). Embryos for injection were

derived from matings of interstrain F1 hybrid females (C57BL6/J and BALB/c) crossed with C57BL6/J males. Transgenic founder mice were backcrossed to C57BL6/J for one to four generations prior to analysis of transgene expression patterns in postnatal offspring (P4 – P28). Genotyping was performed by polymerase chain reaction (PCR) using *nls-lacZ* specific primers 5'-NLS (5'-GAAGACCCCCGCATGGCTCGCGATG-3') and 3'-lacZ (5'-GATCTTCCAGATAACTGCCGTCACTCC-3'). $\beta$-$Actin^{LSL\text{-}nlslacZ}$, $Actin^{Cre}$, $Isl1^{Cre}$ (Srinivas et al., 2001) and $Lim3^{Cre}$ (Sharma et al., 1998) mouse strains have been described previously.

## HISTOLOGY AND IMMUNOHISTOCHEMISTRY

β-galactosidase staining was performed as described previously (Mombaerts et al., 1996). Antibodies used were: rabbit anti-CGRP (Chemicon), rabbit anti-GFP, IgG fraction (Molecular Probes), sheep anti-GFP (Biogenesis), guinea pig anti-Isl1 (Arber et al., 2000), rabbit anti-Ret (IBL). IB4 conjugated to Alexa Fluorescein 488 (Molecular Probes) was used at 4 μg/ml. Cryostat sections were processed for immunohistochemistry as previously described (Arber et al., 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes). Images were collected with an Olympus confocal microscope or with a RT-SPOT camera (Diagnostics Instruments) in case of X-gal staining experiments.

# CHAPTER 5

# IDENTIFYING NOVEL MARKER GENES OF DRG NEURONS

## INTRODUCTION

An essential tool for detailed analysis and understanding of nervous system function is the acquisition of knowledge of appropriate temporal and spatial molecular markers, which allow the unique identification of defined neuronal cell types *in vivo*. Knowledge of such markers will ultimately contribute to a better understanding of neuronal subpopulation specific differentiation and function. Moreover, having appropriate markers in hand will allow visualization of neuronal projections of defined neuronal subclasses at high resolution with the help of novel genetic approaches and modern imaging technologies, thereby revealing and unraveling the complex nature of the intertwined neuronal circuits in the mature mammalian nervous system. Hence, the ability to identify and trace specific neuronal circuits greatly relies on the increasing knowledge of molecular markers expressed by specific neuronal subpopulations.

Apart from the rather coarse classification based on selective neurotrophic factor receptor phenotypes, not many marker genes are known that distinguish different subpopulations of DRG sensory neurons at different stages of development. This fact has prompted attempts in the past to identify novel marker genes expressed in DRG sensory neurons based on differential screening approaches (Akopian and Wood, 1995; Friedel et al., 1997; Dong et al., 2001; Nelson et al., 2004). Using high-density oligonucleotide microarrays we performed a differential screen aimed at the identification of novel marker genes expressed specifically in differentiating DRG neurons during embryonic development.

## RESULTS AND DISCUSSION

In order to identify genes previously not known to be expressed in differentiating DRG neurons we performed an Affymetrix microarray based screen focusing on four different developmental time points, namely E12.5, E14.5, E16.5 and P0.5. At each developmental stage, lumbar DRG and spinal cord from a total of 25-40 wild-type embryos were dissected and pooled to isolate total RNA that was then subjected to gene expression profiling using Affymetrix microarray technology. By comparing gene expression values of DRG with spinal cord it was possible to identify genes expressed exclusively within

DRG but not within spinal cord of the same developmental stage. Furthermore, expression profiling across the four different developmental stages allowed determining the specific temporal gene expression profiles of selected candidate genes. Finally, selected candidate genes were analyzed by *in situ* hybridization experiments to validate the overall screening approach and to identify genes with expression patterns restricted to defined DRG sensory neuron subpopulations (data not shown).

## IDENTIFYING NOVEL MARKER GENES OF PROPRIOCEPTIVE DRG NEURONS

The developmental time course analysis of gene expression in DRG versus spinal cord as outlined above was biased towards identification of genes expressed by a majority of DRG neurons, predominantly within TrkA expressing nociceptive and thermoceptive sensory neurons, which comprise about 70% of lumbar DRG neurons.

Therefore, we complemented our temporal analysis with a genetic approach. To this end, we used two mutant mouse strains, which are characterized by a defect in the generation or survival of distinct classes of DRG neurons, respectively. Since we were mostly interested in identifying genes specifically expressed within proprioceptive DRG sensory neurons, we analyzed *TrkC*$^{-/-}$ as well as *neurogenin 1*$^{-/-}$ (*ngn1*$^{-/-}$) embryos.

In absence of TrkC signaling proprioceptive DRG neurons lack trophic support and undergo programmed cell death soon after they have been generated (Klein et al., 1994; Liebl et al., 1997). As a result, *TrkC*$^{-/-}$ embryos specifically lack proprioceptive sensory neurons while almost all other DRG sensory populations are unaffected by the mutation. Therefore, genes exclusively expressed in proprioceptive DRG neurons should be downregulated when compared to wild-type control DRG of the same developmental stage.

The proneuronal gene *ngn1* encodes a basic helix-loop-helix transcription factor that has been shown to regulate neurogenesis within the DRG (Ma et al., 1999). In homozygous *ngn1* mutants, TrkA expressing nociceptive and thermoceptive sensory neurons fail to be generated during the second wave of neurogenesis in the DRG resulting in a dramatic reduction of DRG size. The only neuronal subpopulations that are still generated in *ngn1*$^{-/-}$ DRG are those belonging to the TrkB positive mechanoreceptive and TrkC

positive proprioceptive subclasses, normally comprising about 30% of lumbar DRG neurons. As a result, genes specifically expressed within these two subpopulations should be upregulated in *ngn1*$^{-/-}$ DRG with respect to control DRG. However, since the dramatic neuronal loss in *ngn1* mutants might also affect the normal ratio of sensory neurons to satellite glia, some of the observed changes in gene expression might be due to an altered neuron to glia ratio rather than reflecting true neuronal subpopulation specific gene expression profiles.

When comparing gene expression values from E14.5 *TrkC*$^{-/-}$ DRG and wild-type littermate DRG, 41 genes were statistically significant decreased by more than 1.2 fold (Table 2). Importantly, among these were known proprioceptive marker genes such as *Er81* (Arber et al., 2000) or *carbonic anhydrase II* (*Car2*; Mayeux et al., 1993).

**Table 2:** Selected candidate genes downregulated in E14.5 lumbar *TrkC*$^{-/-}$ DRG

| Transcript | GenBank | Fold change* | wild-type# | *TrkC*$^{-/-}$# | *ngn1*$^{-/-}$# |
|---|---|---|---|---|---|
| Plzf/Zbtb16 | AI467657 | -1.76 | 169.5 | 94.9 | 230.6 |
| **Er81** | **L10426** | **-1.75** | **590.7** | **325.2** | **1511.5** |
| Bssp-3 | D89871 | -1.71 | 844.8 | 482.0 | 1834.9 |
| EST | AW122691 | -1.70 | 799.1 | 450.9 | 1151.1 |
| Fabph1 | AV268900 | -1.63 | 204.4 | 123.2 | 397.5 |
| Vapb | AI842621 | -1.63 | 2738.2 | 1785.5 | 4375.3 |
| Mana2 | X61172 | -1.51 | 432.4 | 286.9 | 730.4 |
| Gpr64 | AI132005 | -1.49 | 2495.3 | 1637.8 | 5098.6 |
| Pthr1 | X78936 | -1.43 | 265.6 | 180.2 | 455.4 |
| Car2 | M25944 | -1.25 | 287.2 | 223.3 | 463.7 |

* Fold changes were calculated by dividing the normalized intensity values of wild-type DRG by the normalized intensity values of *TrkC*$^{-/-}$ DRG.
# Values represent intensity raw data values of E14.5 wild-type, *TrkC*$^{-/-}$ or *ngn1*$^{-/-}$ DRG, respectively.

Similarly, 42 genes were more than 1.5 fold increased in E14.5 *ngn1*$^{-/-}$ DRG compared to wild-type littermate control DRG, amongst them known proprioceptive markers such as *Er81* (data not shown). Interestingly, *TrkC* itself was not identified by either of the two approaches. Expression levels of *TrkC* were too low, with intensity raw data values in the

range of 20, for being in the considerable range in the statistical data analysis, in which the minimum raw data value cut-off had been set to 50.

## GPCRs – NEURONAL MARKERS OF PROPRIOCEPTIVE DRG NEURONS

Selection of candidate genes for further analysis was restricted mainly to genes encoding either transcription factors or cell surface proteins for the following reasons. Transcription factors expressed in specific neuronal subpopulations at the time of neuronal specification are likely to be involved in controlling lineage specific gene expression ensuring correct determination of neuronal subpopulations (Jessell, 2000; Lee and Pfaff, 2001; Bertrand et al., 2002; Shirasaki and Pfaff, 2002). Likewise, it has been demonstrated that transcription factors control important steps during neuronal circuit formation as has been shown for ETS-domain transcription factors in the assembly of the spinal stretch reflex circuitry (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002).
Secondly, many important developmental processes such as correct neuronal migration, axon guidance and branching, target cell recognition as well as synapse formation all rely on expression of defined sets of cell surface molecules, which are essential signaling molecules required for correct neuronal specification during development (Sanes and Lichtman, 2001; Yu and Bargmann, 2001; Dickson, 2002; Huber et al., 2003; Salinas and Price, 2005). Hence, we focused our initial analysis of candidate novel proprioceptive marker genes specifically on cell surface proteins that could potentially involved in the developmental assembly of the monosynaptic stretch reflex circuit.

We identified two members of the G protein-coupled receptor (GPCR) superfamily as novel candidate genes being selectively downregulated in E14.5 DRG of *TrkC*$^{-/-}$ embryos, thus representing potential novel marker genes expressed specifically by proprioceptive sensory neurons (Table 2). GPCRs are seven-transmembrane (7TM) receptors named after the fact that many of them signal through heterotrimeric G proteins, guanine nucleotide regulatory protein complexes composed of $\alpha$ and $\beta\gamma$ subunits that activate downstream effector molecules such as cyclic nucleotide cyclases or phospholipases (Pierce et al., 2002). The GPCR superfamily is by far the largest and most versatile receptor family in the mammalian genome, reflected by the presence of several 100

GPCR encoding genes in the human genome. GPCR ligands are as diverse as the receptors themselves, ranging from ions, and nucleotides, to amines, peptides, proteins, and lipids, or odorants. Several GPCRs are expressed by specific subsets of nociceptive DRG neurons (Hunt and Mantyh, 2001), among these the NPY receptors, the bradykinin receptor, prostaglandin receptors (Donaldson et al., 2001), opiate receptors (Dado et al., 1993; Bao et al., 2003), and the large family of Mas-related GPCRs (Mrgprs) including the sensory neuron-specific GPCRs (SNSRs; Dong et al., 2001; Lembo et al., 2002).

Recently a subfamily of orphan GPCRs of more than 30 members has been identified, which is characterized by long serine/threonine(Ser/Thr)-rich N-terminal extracellular domains that serve as glycosylation sites and are proposed to function in cell adhesion (Stacey et al., 2000; Fredriksson et al., 2003; Bjarnadóttir et al., 2004). Therefore, these GPCRs have been termed adhesion-GPCRs.

One of the GPCRs identified in our screen as a potential marker for proprioceptive DRG neurons was the 1009aa-long mouse epididymis-specific protein 6 (Me6/Gpr64), belonging to the subfamily of adhesion-GPCRs (Table 2). Knockout mice for the *Gpr64* gene, which is localized on the X chromosome, display male infertility (Davies et al., 2004). However, the role of this orphan GPCR in proprioceptive neuronal differentiation has not yet been analyzed. Interestingly, another member of the same subfamily closely related to Gpr64, Gpr56, has been recently linked causally to a human brain cortical malformation called bilateral frontoparietal polymicrogyria (BFPP), suggesting for the first time a role for adhesion-GPCRs in development of the mammalian nervous system (Piao et al., 2004). Moreover, two expressed sequence tags (ESTs) encoding the adhesion-GPCR Gpr124, which contains two LLR (leucine rich repeat) motifs in its extracellular N-terminal domain not present in Gpr56 and Gpr64, have been isolated from DRG (Bjarnadóttir et al., 2004). Thus adhesion-GPCRs might be commonly expressed by different subpopulations of DRG sensory neurons.

The second GPCR identified in the screen, the parathyroid hormone receptor 1 (Pthr1), is a member of a subfamily of GPCRs vaguely related to adhesion-GPCRs termed secretin receptors after the first receptor cloned in this subgroup (Table 2). The secretin subfamily is characterized by a hormone-binding cysteine-containing N-terminal domain, which

partially displays similarity to the extracellular domain of the adhesion-GPCRs. As its name implies, Pthr1 binds parathyroid hormone (Pth) as well as Pth-related peptide (PTHrP). Mice lacking *Pthr1* die at mid-gestation, whereas heterozygous *Pthr1*$^{+/-}$ mice appear phenotypically normal (Lanske et al., 1996). Analysis of conditional *Pthr1* mutant mice, in which *Pthr1* function was specifically eliminated in early differentiating chondrocytes by a Cre-*lox*P based genetic strategy, demonstrated that Pthr1 signaling controls chondrocyte differentiation at multiple steps (Kobayashi et al., 2002). Interestingly, the parathyroid hormone receptor 2 (Pthr2), which is activated by tuberoinfundibular peptide of 39 residues (TIP39) and weakly by Pth, is expressed by a subpopulation of nociceptive DRG neurons (Dobolyi et al., 2002).

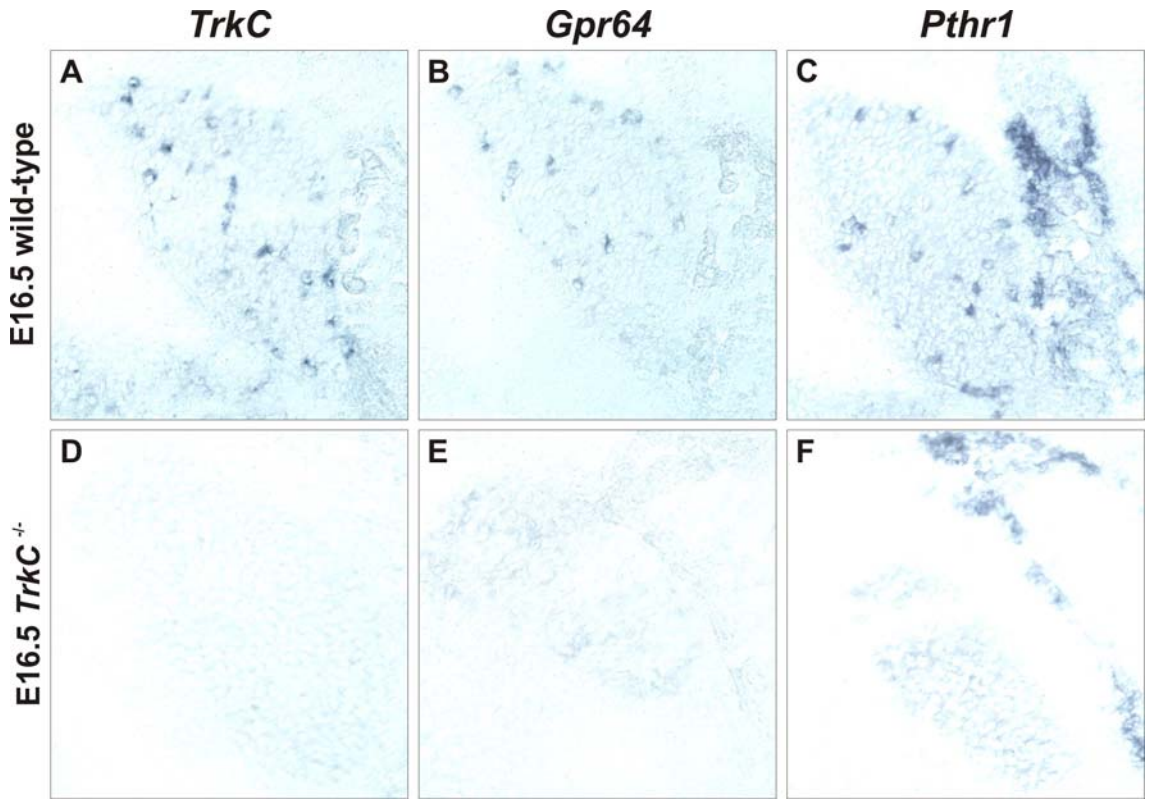


**Figure 21.** Expression of *Gpr64* and *Pthr1* is restricted to proprioceptive sensory neurons

Validation of *GPCR* expression by *in situ* hybridization analysis on sections of E16.5 wild-type (A-C) and *TrkC*$^{-/-}$ (D-F) lumbar DRG using probes for *TrkC* (A, D), *Gpr64* (B, E) or *Pthr1* (C, F). In *TrkC*$^{-/-}$ DRG (D-F) expression of both GPCRs is not detectable, thus confirming their subpopulation specific expression as being restricted to proprioceptive sensory neurons. (Images kindly provided by A. Friese.)

In order to validate gene expression values of novel candidate marker genes identified in the screen *in situ* hybridization experiments were carried out comparing gene expression patterns in wild-type and *TrkC*$^{-/-}$ embryos to determine whether candidate genes were specifically only expressed by proprioceptive DRG neurons and not by other subpopulations of DRG neurons (Figure 21). Gene expression of the two GPCR encoding genes, *Gpr64* and *Pthr1*, was completely abolished in E16.5 *TrkC* mutant DRG, thus confirming that these two GPCRs are expressed selectively by proprioceptive DRG neurons only.

Moreover, Gpr64 colocalizes with the cytoplasmic $Ca^{2+}$ binding protein parvalbumin (PV), a well-established proprioceptive sensory neuron marker (Figure 23B, D). In contrast to parvalbumin, whose expression in proprioceptive sensory neurons starts only around E14, Gpr64 expression was already detectable in E12.5 brachial and lumbar DRG (Figure 22A-D).

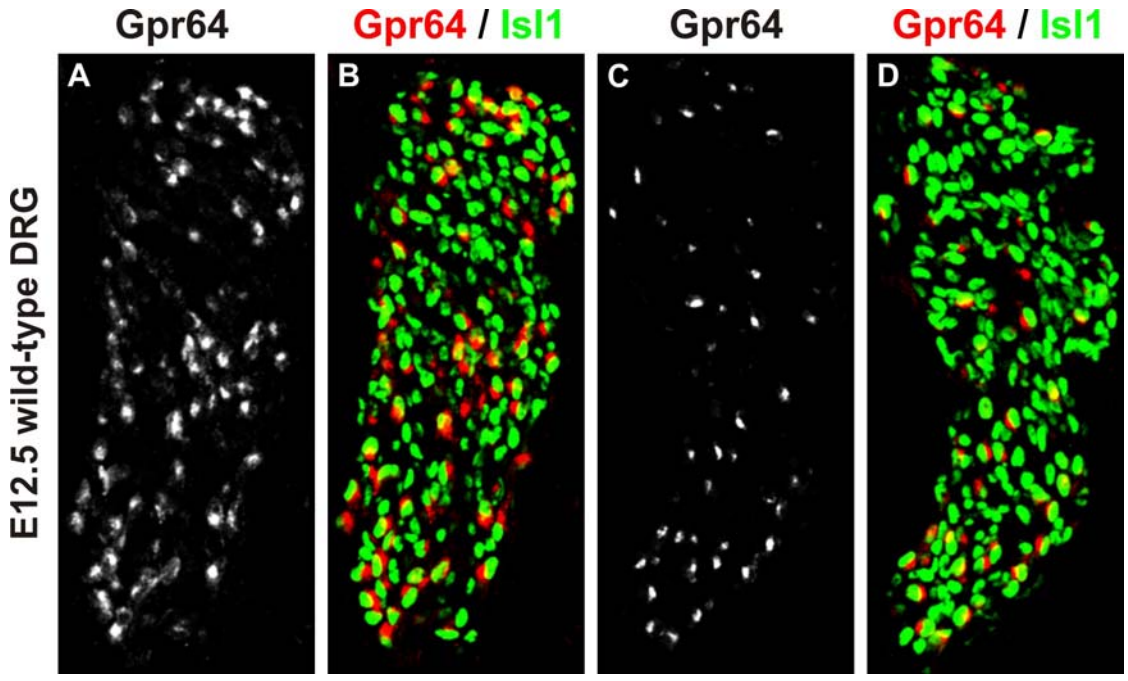


**Figure 22.** Gpr64 expression is detectable in E12.5 DRG sensory neurons

Immunohistochemical analysis of Gpr64 expression on sections of E12.5 wild-type brachial (A, B: red) and lumbar DRG (C, D: red). Coexpression with the postmitotic neuronal marker Isl1 (B, D: green) demonstrates exclusively neuronal expression of Gpr64.

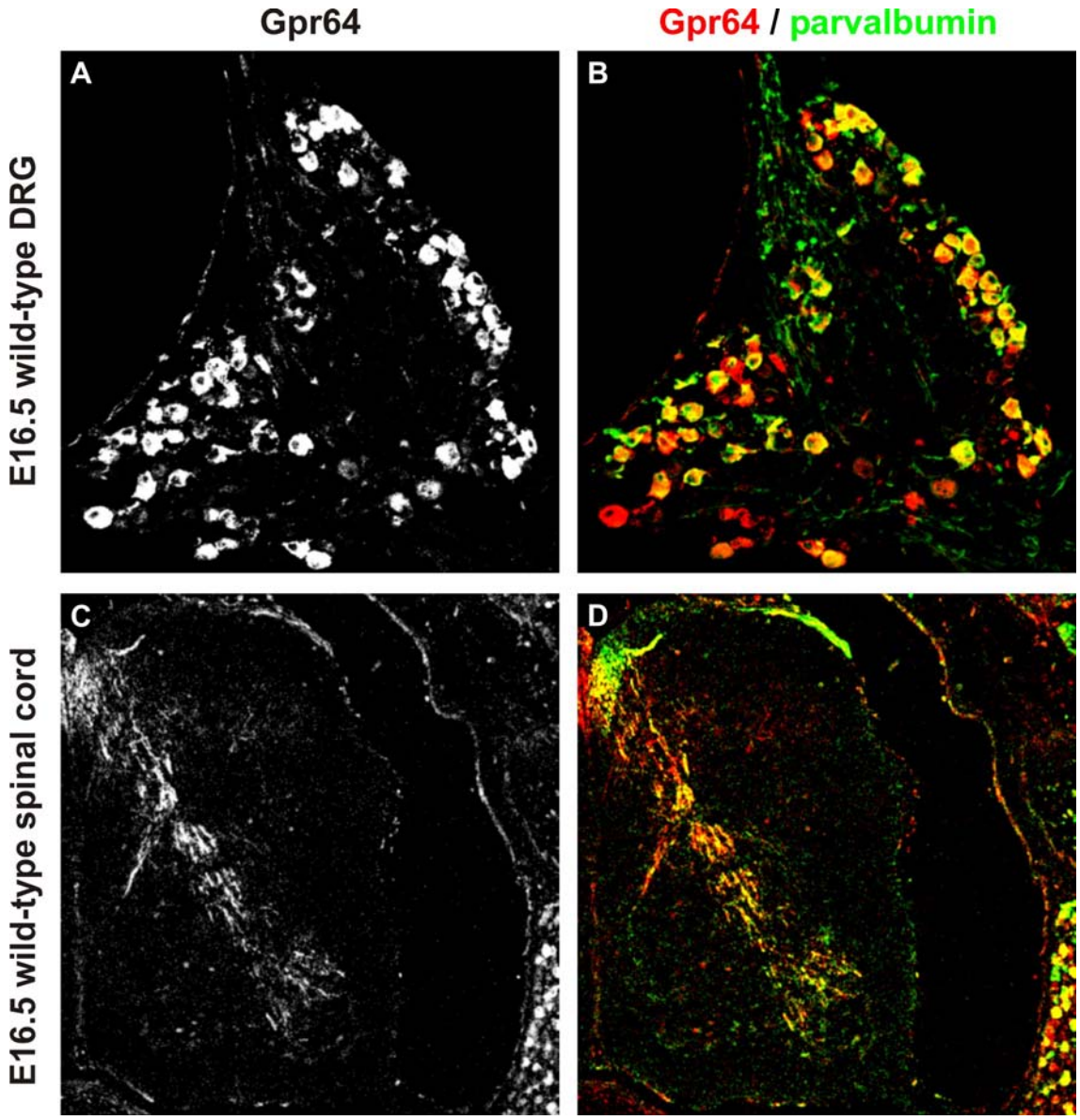


**Figure 23.** Characterization of Gpr64 expression in proprioceptive DRG neurons

Validation of Gpr64 expression in proprioceptive DRG neurons by immunohistochemical analysis on sections of E16.5 wild-type brachial DRG (A, B: red), spinal cord (C, D: red). Note colocalization of Gpr64 with the $Ca^{2+}$ binding protein parvalbumin (B, D: green), a proprioceptive marker, in cell bodies (B) and central axon collaterals (D) of proprioceptive DRG sensory neurons.

Therefore, while presumably many proprioceptive neuron specific genes were missed by our approach, since only 41 genes were identified as being significantly downregulated in E14.5 *TrkC*$^{-/-}$ DRG, it is likely that many of the identified genes indeed represent novel proprioceptive marker genes, as could be shown for the two GPCR encoding genes,

*Gpr64* and *Pthr1*. In future experiments it will be exciting to determine the consequences of loss of either of the two newly identified GPCRs, Gpr64 and Pthr1, with respect to proprioceptive sensory neuron differentiation.

# METHODS

## RNA EXTRACTION

Lumbar DRG and spinal cord covering the entire lumbar region from L1 to L6 were dissected in ice-cold Leibovitz's 15 (L15) medium from mouse embryos of different developmental stages and genotypes. Isolated spinal cords were cut horizontally to obtain dorsal and ventral parts with the exception of spinal cords isolated from E12.5 embryos, which were left intact. Isolated DRG and spinal cords were stored in RNAlater (Ambion) at –20°C until pooled prior to total RNA extraction. Depending on the different developmental stages, lumbar DRG from 25-40 embryos (corresponding to 300-480 DRG) or lumbar spinal cords from 8-16 embryos were pooled prior to total RNA extraction. Total RNA was isolated using TRIzol reagent (GibcoBRL, Life Technologies) and purified using the RNeasy Mini kit (Qiagen) yielding final concentrations of total RNA of 1-2 μg/μl of high RNA quality ($A_{260}/A_{280} > 1.9$).

## MICROARRAY GENE EXPRESSION ANALYSIS

Affymetrix microarray experiments were performed at the microarray service facility of the Friedrich Miescher Institute for Biomedical Research, Basel, according to the manufacturer's instructions using 10 μg total RNA. First- and second-strand DNA synthesis reactions were performed by using the Superscript Choice System (Invitrogen) followed by in vitro transcription (Enzo Diagnostics) using biotin-labeled dNTPs to generate biotinylated cRNA as for the process recommended by Affymetrix. cRNA was fragmented and hybridized to U74Av2 and U47Bv2 GeneChips (Affymetrix), which were scanned in an Affymetrix Agilent 2500 scanner. Expression values were estimated using Affymetrix Microarray Suite v4. Data analysis was performed with GeneSpring 4.1

(Silicon Genetics) software, filtering for expression raw data values of at least 50 and fold changes larger than 1.5 fold (or 1.2 fold in case of *TrkC* mutant analysis) between two compared conditions. Changes were considered significant if they had an Affymetrix change call of increase or decrease ($p < 0.003$) and passed a *t*-test ($p < 0.05$) with a Benjamini and Hochberg multiple testing correction.

## *IN SITU* HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

For *in situ* hybridization analysis, cryostat sections of fresh frozen tissue were hybridized with digoxigenin labeled antisense riboprobes directed against rat *TrkC* (gift from L. F. Parada), mouse *Gpr64* (BC023772) or mouse *Pthr1* (BC051981) as previously described (Schaeren-Wiemers and Gerfin-Moser; 1993). Antibodies used were: rabbit anti-Gpr64 (gift from C. Osterhoff; Obermann et al., 2003), guinea pig anti-Isl1 (Arber et al., 2000) and rabbit anti-parvalbumin (SWANT). Cryostat sections were processed for immunohistochemistry as previously described (Arber et al., 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes). Images were taken with an Olympus confocal microscope or for *in situ* hybridization experiments with a RT-SPOT camera (Diagnostics Instruments).

# CHAPTER 6

# RUNX TRANSCRIPTION FACTORS: REGULATORS OF CELLULAR DIFFERENTIATION

# INTRODUCTION

One interesting class of transcription factors identified in the screen outlined in the previous chapter (data not shown) were the runt related transcription factors Runx1 and Runx3, which share a conserved 128aa-long S-type immunoglobulin fold DNA binding domain called runt domain (RD) with the *Drosophila* segmentation gene *runt* (*run*). Runx transcription factors, also referred to as acute myeloid leukemia (AML) or polyoma enhancer-binding protein 2α (PEBP2α) family members (Van Wijnen et al., 2004), represent the α-subunits of the heterodimeric core binding factor (Cbf) complex composed of a DNA binding α-subunit and a common β-subunit, called Cbfβ, which by itself lacks DNA binding activity and a nuclear localization signal (NLS).

In mammals three α-subunit encoding genes have been identified (Table 3), which have been termed *Runx1* (*Cbfα2*/*Aml1*/*Pepb2αB*), *Runx2* (*Cbfα1*/*Aml3*/*Pepb2αA*), and *Runx3* *Cbfα3*/*Aml2*/*Pepb2αC*). Interaction with the partner subunit Cbfβ, which binds directly to the conserved runt DNA binding domain, is required for stabilization of the α-subunits on the DNA target, as all three Runx factors only weakly bind to a common conserved DNA consensus sequence, 5'-R/TACCACA-3' (Nagata et al., 1999). Hence, similar to ETS transcription factors, Runx proteins also rely on a complex regulatory network of various inter- and intramolecular interactions mediating target gene promoter specificity, which is highly dependent on the respective cellular context (Ito, 1999; Blyth et al., 2005). Moreover, Runx transcription factor activity and protein stability are regulated by post-translation modifications such as phosphorylation (Tanaka et al., 1996; Le et al., 1999; Wee et al., 2002), acetylation (Yamaguchi et al., 2004; Jin et al., 2004) and ubiquitination (Tintut et al., 1999).

Interestingly, depending on the interacting partner proteins and the respective promoter context Runx transcription factors can either lead to transcriptional activation or repression of target genes. Target gene repression can be mediated for example by recruitment of the Groucho/transducin-like enhancer of split (TLE) transcriptional corepressors, which bind to a conserved motif, VWRPY, present at the C-terminus of all Runx factors (Aronson et al., 1997; Levanon et al., 1998; Javed et al., 2000).

**Table 3:** Functions of mouse runt related transcription factors*

| Gene | Alternative gene names | Proposed essential function | Mouse (–/–) phenotype |
|---|---|---|---|
| *Runx1* | *Aml1/Cbfα2/Pebp2αB* | definitive fetal liver hematopoiesis | embryonic lethal due to absence of fetal liver hematopoiesis |
| *Runx2* | *Aml3/Cbfα1/Pepb2αA* | osteoblast differentiation, bone formation | lethal at birth due to respiratory failure |
| *Runx3* | *Aml2/Cbfα3/Pepb2αC* | proprioceptive sensory neuron differentiation | perinatal lethal |

* Adapted from Lund and van Lohuizen, 2002.

Analysis of mouse mutants for the *Runx* genes demonstrated that all three Runx transcription factors function as important regulators of cell fate determination during embryonic development by controlling fundamental steps of cellular differentiation and cell lineage specification (Table 3).

Homozygous deletion of *Runx1* causes embryonic lethality by midgestation (E11.5-12.5) due to lack of definitive fetal liver hematopoiesis, demonstrating that Runx1 is essential for development of the hematopoetic lineages (Okuda et al., 1996; Wang et al., 1996a). Moreover, the *Runx1* locus is one the most frequent targets of chromosomal translocations leading to occurrence of acute myeloid leukemias in humans (Nucifora and Rowley, 1995).

Interestingly, generation and analysis of conditional *Runx1* mutant mice has recently revealed that Runx1 also plays important functions in the CNS where it is involved in differentiation of olfactory receptor neuron progenitors as well as in postmitotic motor neurons of the hindbrain (Theriault et al., 2004; 2005). While loss of Runx1 function does not interfere with early motor neuron specification it is critically required for later steps of differentiation, as in absence of Runx1 activity hindbrain motor neurons undergo increased programmed cell death. Moreover, Runx1 inactivation also leads to early loss of selected classes of nociceptive and mechanoreceptive sensory neurons in trigeminal and vestibulocochlear ganglia, which normally express Runx1 (Theriault et al., 2004). In contrast, subpopulations of Runx1 positive somatic MMCm and LMCm motor neurons

present in the cervical spinal cord do not rely on Runx1 for survival (Theriault et al., 2004).

*Runx2* deficient mice die at birth with severe defects in bone development due to failure of osteoblast differentiation, partially reminiscent of a human skeletal malformation associated with *RUNX2* haploinsufficiency called cleidocrancial dysplasia syndrome (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Inada et al., 1999; Kim et al., 1999a).

Runx3 has been shown to play important roles in proprioceptive DRG sensory neuron differentiation, in thymopoiesis and in dendritic cell function (Inoue et al., 2002; Levanon et al., 2002; Taniuchi et al., 2002a; Woolf et al., 2003; Fainaru et al., 2004). From the first analysis of *Runx3* knockout mice it was concluded that Runx3 acts as a tumor suppressor, since *Runx3* deficiency resulted in gastric epithelial hyperplasia due to loss of sensitivity to the growth-inhibitory and apoptosis-inducing activities of transforming growth factor β (TGFβ; Li et al., 2002). However, the interpretation of this phenotype has been controversial, as it has been proposed that the gastric phenotype reflects a secondary consequence of chronic immune-mediated inflammation (Bae and Ito, 2003; Levanon et al., 2003; Brenner et al., 2004). Moreover, there has been conflicting evidence concerning the proposed role for Runx3 in proprioceptive DRG sensory neuron differentiation. The first publication addressing this issue showed that in absence of Runx3 function proprioceptive DRG sensory neurons fail to maintain TrkC expression and as a consequence undergo programmed cell death, reminiscent of the reported *TrkC* knockout phenotype (Klein et al., 1994; Liebl et al., 1997; Levanon et al., 2002). Thus, *Runx3* knockout mice display a highly ataxic phenotype due to lack of sensory-motor connectivity.

While the ataxic phenotype was also described in the second independent *Runx3* knockout study, the underlying cause appears to be fundamentally different, as no effect on maintenance of TrkC expression was reported (Inoue et al., 2002). In contrast, proprioceptive DRG neurons survive and express known markers of proprioceptive differentiation such as Er81 and parvalbumin. Nevertheless, innervation of central and peripheral targets was highly abnormal, as proprioceptive afferents fail to invade their

normal central targets within the spinal cord and to establish or maintain normal peripheral target innervation, thus explaining the strong ataxic phenotype.

Finally, disruption of the *Cbfβ* gene phenocopies loss of *Runx1* function, thus reflecting the absolute requirement of Cbfβ as a stabilizing partner subunit for Runx1 (Sasaki et al., 1996; Wang et al., 1996b). Similarly, Cbfβ is essential for Runx2 function during bone formation as was demonstrated by lineage-specific rescue of the hematopoetic defects observed in *Cbfβ*$^{-/-}$ mice (Kundu et al., 2002; Miller et al., 2002; Yoshida et al., 2002). While the specific requirement of Cbfβ for Runx3 activity has not yet been demonstrated *in vivo*, *in vitro* data suggest that also Runx3 activity depends on the presence of Cbfβ in analogy to its role in mediating stabilization of the two other Runx family members.

To characterize Runx transcription factor expression in more detail we raised polyclonal antisera against Runx1 and Runx3 allowing further characterization of their expression pattern in developing mouse DRG sensory neurons. While Runx3 is selectively expressed in TrkC positive proprioceptive DRG neurons, Runx1 is expressed in a subset of TrkA positive cutaneous DRG neurons. In addition, using RT-PCR we found that DRG sensory neurons appear to predominantly express the shorter N-terminal Runx isoforms derived from the conserved proximal P2 promoters of the *Runx1* and *Runx3* genes.

## RESULTS AND DISCUSSION

### RUNX TRANSCRIPTION FACTORS ARE EXPRESSED IN DISTINCT SUBPOPULATIONS OF DRG NEURONS

To confirm previous reports about Runx transcription factor expression in differentiating DRG sensory neurons (Levanon et al., 2001a) we analyzed Runx1 and Runx3 expression in E16.5 lumbar DRG of wild-type embryos. Runx1 expression was restricted to many small diameter sensory neurons (Figure 24A, C, D, E) and there was no coexpression detectable of Runx1 and the $Ca^{2+}$ binding protein parvalbumin (Figure 24E), a known marker protein of proprioceptive DRG neurons. In contrast, Runx3 expression was

restricted to large diameter sensory neurons representing proprioceptive DRG neurons, as pronounced coexpression of Runx3 and parvalbumin was observed (Figure 24H).

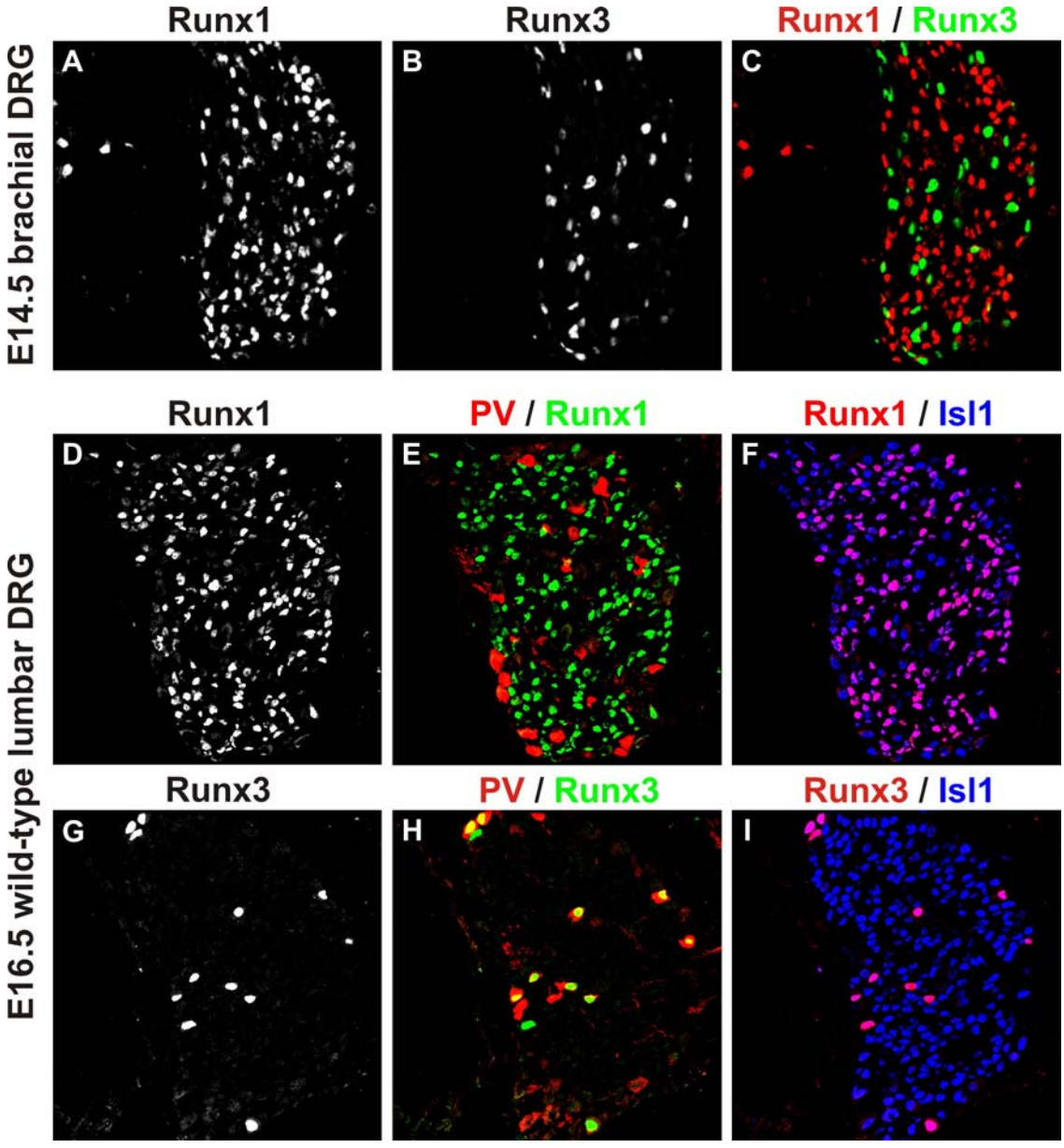


**Figure 24.** Expression of Runx transcription factors in distinct populations of DRG neurons

Immunohistochemical analysis of Runx1 and Runx3 expression on sections of E14.5 wild-type brachial DRG (A-C) and E16.5 wild-type lumbar DRG (D-I). No colocalization of Runx1 and Runx3 is detectable (C). (D-F) Triple labeling experiment to detect Runx1 (E: green; F: red), parvalbumin (PV; E: red) and Isl1 (F: blue). (G-I) Triple labeling experiment to detect Runx3 (H: green; I: red), parvalbumin (PV; H: red) and Isl1 (I: blue). Note coexpression of Runx3 and parvalbumin (H), but absence of coexpression of Runx1 and parvalbumin (E).

Moreover, even at earlier developmental stages Runx1 and Runx3 segregated into distinct sensory neuron subpopulations, as there was no colocalization of the two Runx transcription factors observable when analyzed at E14.5 (Figure 24C). Thus, Runx1 and Runx3 transcription factors are expressed by different subpopulations of DRG sensory neurons. While Runx1 is selectively expressed in a subset of TrkA positive cutaneous sensory neurons (data not shown), Runx3 expression is restricted to proprioceptive DRG neurons, also reflected by the loss of Runx3 expression in E17.5 *TrkC*$^{-/-}$ embryos, in which proprioceptive sensory neurons undergo programmed cell death (data not shown).

## DRG NEURONS MAINLY EXPRESS *RUNX* PROXIMAL PROMOTER ISOFORMS

A common feature of the vertebrate *Runx* genes is the use of alternative transcription start sides from two conserved promoters, a distal promoter P1 and a proximal promoter P2, giving rise to two alternative Runx isoforms differing only in the N-terminal amino acid composition prior to the conserved runt DNA binding domain (Levanon and Groner, 2004). While some attempts have been undertaken to characterize the individual Runx isoforms in more detail, conclusive evidence is still missing to which extend the two promoter isoforms differ with respect to their developmental regulation and expression or their specific functions (Ghozi et al., 1996; Bangsow et al., 2001; Levanon et al., 2001b; Rini and Calabi, 2001). The proximal P2 promoter is located within a large CpG island, which is not the case for the distal P1 promoter. Interestingly, the distal P1 promoter contains conserved DNA binding sides for ETS transcription factors. Moreover, both promoters have been shown to contain several dispersed Runx DNA binding sequences, indicating that *Runx* genes might be subject to auto- or cross-regulatory transcriptional control by other Runx family members.

In order to determine which *Runx* promoter isoforms are expressed in differentiating DRG sensory neurons and whether their expression would be developmentally regulated reverse transcription-PCR (RT-PCR) experiments were performed using promoter isoform specific oligonucleotide primers for *Runx1* and *Runx3* transcripts (Figure 25). While transcripts encoding both *Runx1* promoter isoforms were detectable in cDNA prepared from17.5 DRG, the P2 derived proximal promoter isoform appears to be the

predominant isoform, as the P1 derived promoter isoform was only very weakly detectable (Figure 25A). Interestingly, in E12.5 DRG only the P2 derived proximal promoter isoform could be detected. However, since *Runx1* only starts to be expressed in DRG from E12.5 onwards (data not shown), it cannot be excluded that the amount of P1 derived transcripts was simply too low to be detectable at this early developmental time point.

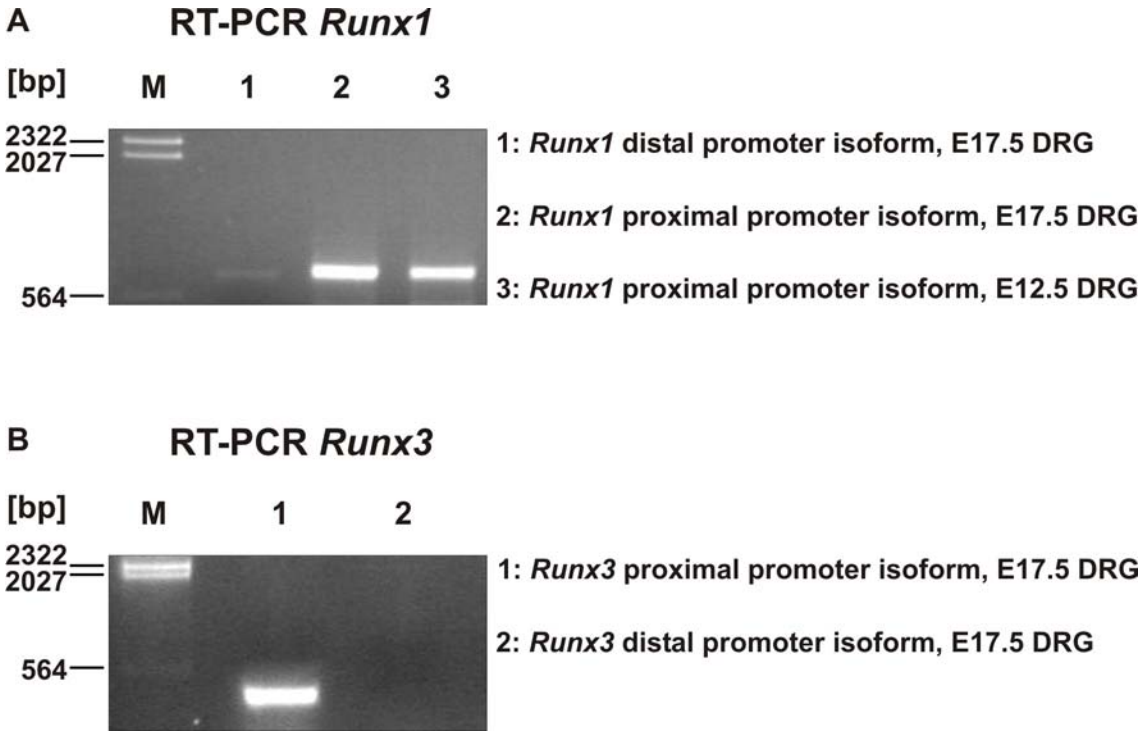


**Figure 25.** Expression of distinct isoforms of *Runx* transcripts in DRG sensory neurons

RT-PCR analysis of *Runx1* (A) or *Runx3* (B) promoter specific isoforms in embryonic DRG. **(A)** In E17.5 DRG both *Runx1* promoter isoforms are detectable. However, the distal P1 promoter derived isoform (lane 1) appears to be expressed at lower levels than the proximal P2 promoter derived isoform (lane 2). In E12.5 DRG only proximal P2 promoter derived *Runx1* transcripts are detectable (lane 3). **(B)** In E17.5 lumbar DRG only proximal P2 promoter derived *Runx3* transcripts are detectable (lane 1), whereas distal P1 promoter derived transcripts are not (lane 2). Abbreviations: M = DNA Λ-marker.

In contrast to *Runx1* the distal P1 promoter derived isoform of *Runx3* was neither detectable in E12.5 DRG (data not shown), nor in lumbar E17.5 DRG, whereas proximal P2 promoter derived transcripts were prominently expressed both in E12.5 DRG (data not shown) and in E17.5 DRG (Figure 25B). Importantly, in control experiments it was however possible to amplify both *Runx3* promoter isoforms from endogenous transcripts

present in adult thymus (data not shown). Nevertheless, it cannot be excluded that the method used was not sensitive enough for a detection of small amounts of P1 promoter derived *Runx3* transcripts, especially since *Runx3* is only expressed in a minority of DRG sensory neurons, namely in proprioceptive neurons, which form only 10-20% of all DRG neurons.

Recently, it was reported that cross-regulatory repression of the *Runx1* distal P1 promoter occurs in human B cells by Runx3 expressed from the proximal P2 promoter (Spender et al., 2005). These findings suggest that under certain cellular conditions P2 promoter derived Runx isoforms are dominant over P1 derived Runx isoforms, contributing to the selective expression of P2 promoter derived isoforms. It is thus tempting to speculate that the distal P1 and proximal P2 promoter derived *Runx1* isoforms detected by RT-PCR in E17.5 DRG are not coexpressed within the same neurons, but rather mark selective subpopulations of cutaneous DRG neurons.

## METHODS

### IMMUNOHISTOCHEMISTRY

Antibodies used were: guinea pig anti-Runx1 and rabbit anti-Runx1 (generated against a mouse Runx1 peptide: GRASGMTSLSAELSSRL), rabbit anti-Runx3 (generated against a 6xHis tagged carboxy-terminal fusion protein of Runx3; amino acids 187-415), rabbit anti-Runx3 (generated against a mouse Runx3 peptide: MSAAFPYSATPS). Cryostat sections were processed for immunohistochemistry as previously described (Arber et al, 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes). Images were collected with an Olympus confocal microscope.

## RT-PCR

RT-PCR was performed according to the manufacturer's instructions (Promega) using total RNA extracts from E12.5 or E17.5 DRG of wild-type mouse embryos and the

following oligonucleotides: 5'-GTGCGTTTTCGAAAGGAAACG-3' (distal *Runx1* P1 promoter isoform specific forward primer), 5'-GTCTAGTAGGAGCTGTTTTCAGG3' (proximal *Runx1* P2 promoter isoform specific forward primer), 5'-GCAGTGGAGTGGTTCAAGGAGG-3' (*Runx1* reverse primer), 5'-GAACCCAACCCCCTGAGGCCG-3' (distal *Runx3* P1 promoter isoform specific forward primer), 5'-CGCCCTGACGGCCGCGGCATG-3' (proximal *Runx3* P2 promoter isoform specific forward primer) and 5'-GCGGAGTAGTTCTCATCATTG-3' (*Runx3* RD reverse primer). All RT-PCR products were subcloned and verified by DNA sequencing.

# CHAPTER 7

## THE STATUS OF RUNX3 EXPRESSION BY DEVELOPING SENSORY NEURONS DETERMINES NEUROTROPHIC FACTOR RECEPTOR PHENOTYPE

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Manuscript submitted.

## SUMMARY

The expression of distinct neurotrophic factor receptors in defined subpopulations of sensory neurons in the dorsal root ganglion (DRG) is associated with the establishment of unique functional properties and patterns of connectivity during embryonic development, but the molecular mechanisms that underlie the selectivity of receptor choice remain unclear. We have used genetic manipulations in the mouse embryo to determine whether the status of expression of the runt domain transcription factor Runx3 by sensory neurons controls the segregation of TrkB, TrkC and Ret receptor expression into discrete sensory neuron subclasses. Our findings indicate that Runx3 promotes the differentiation of TrkC positive proprioceptive sensory neurons through the coordinate extinction of a TrkB positive phenotype.

## INTRODUCTION

The initial processing of somatosensory information from the periphery to the spinal cord is mediated by primary sensory neurons located in the dorsal root ganglion (DRG; Scott, 1992). Distinct subclasses of DRG neurons convey different sensory modalities, but the molecular mechanisms that regulate sensory neuron diversity remain unclear. Specific sensory neuron subtypes in the DRG express transmembrane receptor tyrosine kinases that transduce signals from neurotrophic factors (Bibel and Barde, 2000; Huang and Reichardt, 2003) and there is a tight correlation between the expression of specific neurotrophic factor receptors and the sensory modality and central projection pattern of primary sensory neurons (Scott, 1992; Bibel and Barde, 2000). Resolving the mechanisms that underlie neurotrophic factor receptor choice has therefore emerged as a central problem in defining the logic that drives the diversification and function of sensory neurons in the DRG (Markus et al., 2002b).

Two major classes of neurotrophic factor receptors delineate sets of newly generated DRG neurons. Trk kinases serve as receptors for the neurotrophins NGF, BDNF and NT3, and define three major subclasses of DRG sensory neurons. TrkA is expressed by nociceptive and thermoceptive afferents, TrkB is expressed by a subpopulation of

cutaneous mechanoreceptive afferents and TrkC is expressed by proprioceptive afferents (Bibel and Barde, 2000; Huang and Reichardt, 2003). In addition, Ret, a receptor tyrosine kinase that transduces signaling by glial cell line derived neurotrophic factor (GDNF) family proteins, defines a subpopulation of DRG neurons at early stages, although the sensory modality that they transduce has not been defined (Molliver et al., 1997; Baudet et al., 2000). The selection of neurotrophic factor receptor subtype has a critical influence on later aspects of sensory neuron differentiation. By gating responses to trophic ligands, neurotrophic factor receptor expression is essential for the survival of specific sensory neuron subsets (Bibel and Barde, 2000; Huang and Reichardt, 2003). Neurotrophin signaling also plays an important role in the control of axon target invasion, and in the acquisition of mature phenotypic traits, notably the expression of peptidergic neurotransmitters (Markus et al., 2002b; Patel et al., 2000; Patel et al., 2003; Hellard et al., 2004; Hippenmeyer et al., 2004). More recently, the selectivity of TrkA and TrkC signaling in DRG neurons has been suggested to play an instructive role in determining the phenotypic character of cutaneous and proprioceptive afferents (Moqrich et al., 2004).

How is the expression of specific neurotrophic factor receptors by sensory neurons controlled? Lineage tracing experiments in the chick embryo suggest that sensory neuron progenitors are not committed to a specific subtype fate (Frank and Sanes., 1991). Instead, the selectivity of sensory phenotype revealed by neurotrophin receptor expression, emerges gradually after cell cycle exit. Some DRG neurons have been shown to coexpress TrkB and TrkC receptors shortly after cell cycle exit (Farinas et al., 1998). Together, these observations raise the question of the molecular mechanism underlying neuronal subtype specification within the DRG, and in particular the mechanisms by which the selectivity of expression of neurotrophic factor receptors is achieved.

Several transcription factors have been implicated in the generation and differentiation of subpopulations of DRG neurons. The generation of DRG sensory neurons is controlled in progenitor cells by the combinatorial activities of Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2), two proneural transcription factors of the basic helix-loop-helix class (Ma et al., 1999). Most TrkC positive and TrkB positive DRG neurons appear to be derived from Ngn2 precursors whereas *Ngn1* is required to generate the majority of TrkA positive

afferents (Ma et al., 1999). Once generated, two major subpopulations of DRG sensory neurons express the runt related transcription factors Runx1 and Runx3 (Inoue et al., 2002; Levanon et al., 2002). Runx3 expression has been reported to be confined to TrkC positive proprioceptive afferents, and consistent with a role for Runx3 in proprioceptive afferent differentiation, postnatal *Runx3* mutant mice exhibit defective intraspinal trajectories and motor behavioral defects (Inoue et al., 2002; Levanon et al., 2002). In contrast, Runx1 is expressed by TrkA positive DRG neurons (Inoue et al., 2002; Levanon et al., 2002). To date, no association of Runx transcription factor expression with TrkB positive DRG neurons has been reported (Levanon et al., 2002). These findings raise the possibility that the differential expression of Runx transcription factors in DRG neurons controls the differentiated phenotype of sensory neuron subsets, as defined by expression of individual neurotrophic factor receptors.

Here we define one aspect of the developmental control of neurotrophic factor receptor expression in DRG sensory neuron subtypes. Using gain and loss of function genetic manipulations in the mouse, we show that Runx3 expression in prospective proprioceptive sensory neurons is essential to promote the transition from a hybrid TrkB+/TrkC+ neuronal phenotype to a sensory neuron subpopulation that expresses TrkC alone. Ectopic Runx3 also induces TrkC expression in Ret positive DRG neurons, but the transition from a TrkB+/Ret+ to a Ret positive phenotype is not dependent on Runx3 expression. Together, our findings indicate that the status of Runx3 expression contributes to the emergence of a proprioceptive sensory phenotype by maintaining TrkC and suppressing TrkB neurotrophic factor receptor expression.

## RESULTS

### RUNX3 EXPRESSION IS CONFINED TO TRKC+, BUT NOT TRKB+ OR RET+ DRG NEURONS

To study whether the status of Runx3 expression participates in the segregation of sensory neuron subpopulations, as defined by the expression of distinct neurotrophic

factor receptors we examined the profile of neurotrophic factor receptor expression soon after sensory neuron generation. We focused on neurons that expressed TrkC, TrkB and Ret, since these neuronal populations are generated shortly before the onset of Runx3 expression and prior to the generation of the majority of TrkA positive neurons (Ma et al., 1999).

In lumbar level sensory neurons, the onset of TrkC expression preceded slightly that of TrkB and Ret expression (Figure 27K), but by E11.5, all three proteins were detected (Figure 26A-D; data not shown). The identity of the early Ret positive sensory neurons is intriguing. A population of Ret positive DRG neurons has been documented at late embryonic stages, and this population derives from TrkA positive sensory neurons (Molliver et al., 1997). However, several lines of evidence indicate that early Ret positive DRG neurons do not derive from TrkA positive DRG neurons. Most critically, these Ret positive neurons persist in *TrkA*$^{-/-}$ embryos as well as in *Ngn1* mutants (Figure 27G, H), where the generation of most TrkA positive sensory neurons is abolished (Ma et al., 1999).

At early stages, coexpression of TrkC, TrkB and Ret was observed. The incidence of hybrid TrkB+/TrkC+ neurons dropped from about 40% at E11.5 to approximately 10% at E12 (Figure 26C, D, G, H), in agreement with previous observations (Farinas et al., 1998). Strikingly, there was also pronounced coexpression of Trk with Ret: at E12, about 80% of Ret positive neurons coexpressed TrkB (Figure 26J, L), whereas no coexpression between TrkC and Ret was detected (Figure 26K, L). To assess whether the hybrid TrkB+/TrkC+ and TrkB+/Ret+ neurons observed at E12 persisted at later developmental stages, we analyzed DRG neurons at E14.5. We detected no coexpression of TrkB and TrkC, and only few Ret positive DRG neurons coexpressed any of the three Trk receptors (Figure 27A-C; data not shown). Together, these findings suggest that hybrid TrkB+/TrkC+ and TrkB+/Ret+ neurons represent an early and transient population of sensory neurons.

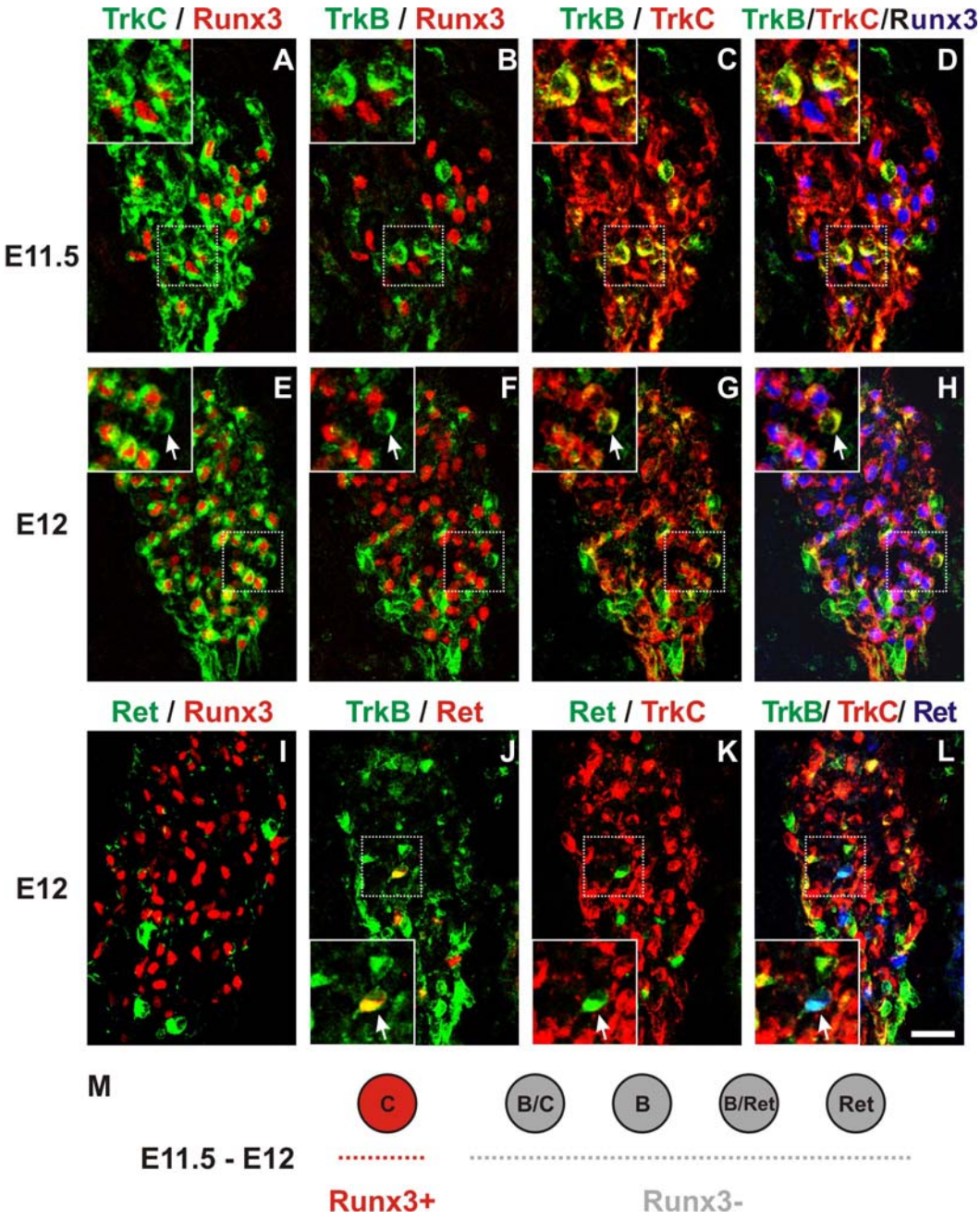


**Figure 26.** Runx3 expression is restricted to pure TrkC DRG sensory neurons

Immunohistochemical analysis of E11.5 (A-D) and E12 (E-L) lumbar level DRG. Insets depict area of dotted box shown at higher magnification. **(A-H)** Triple labeling experiment to detect TrkC (A, E: green; C, D, G, H: red), TrkB (B-D, F-H: green) and Runx3 (A, B, E, F: red; D, H: blue). Note absence of Runx3 expression in hybrid TrkB+/TrkC+ sensory neurons (arrows in E-H).
**(I)** Double labeling experiment to detect Ret (green) and Runx3 (red). **(J-L)** Triple labeling experiment to detect TrkB (J, L: green), TrkC (K, L: red) and Ret (J: red; K: green; L: blue). Note colocalization of TrkB and Ret, but absence of colocalization between TrkC and Ret (arrows).
**(M)** Summary diagram depicting five subpopulations of lumbar DRG sensory neurons expressing TrkB, TrkC and Ret at E11.5-E12 and their status of Runx3 expression (Runx3+: red; Runx3-: grey): TrkC+, TrkC+/TrkB+, TrkB+, Ret+/TrkB+, Ret+. Scale bar: (A-D) = 24 μm; (insets A-D) = 14 μm; (E-L) = 30 μm; (insets E-L) = 17 μm.

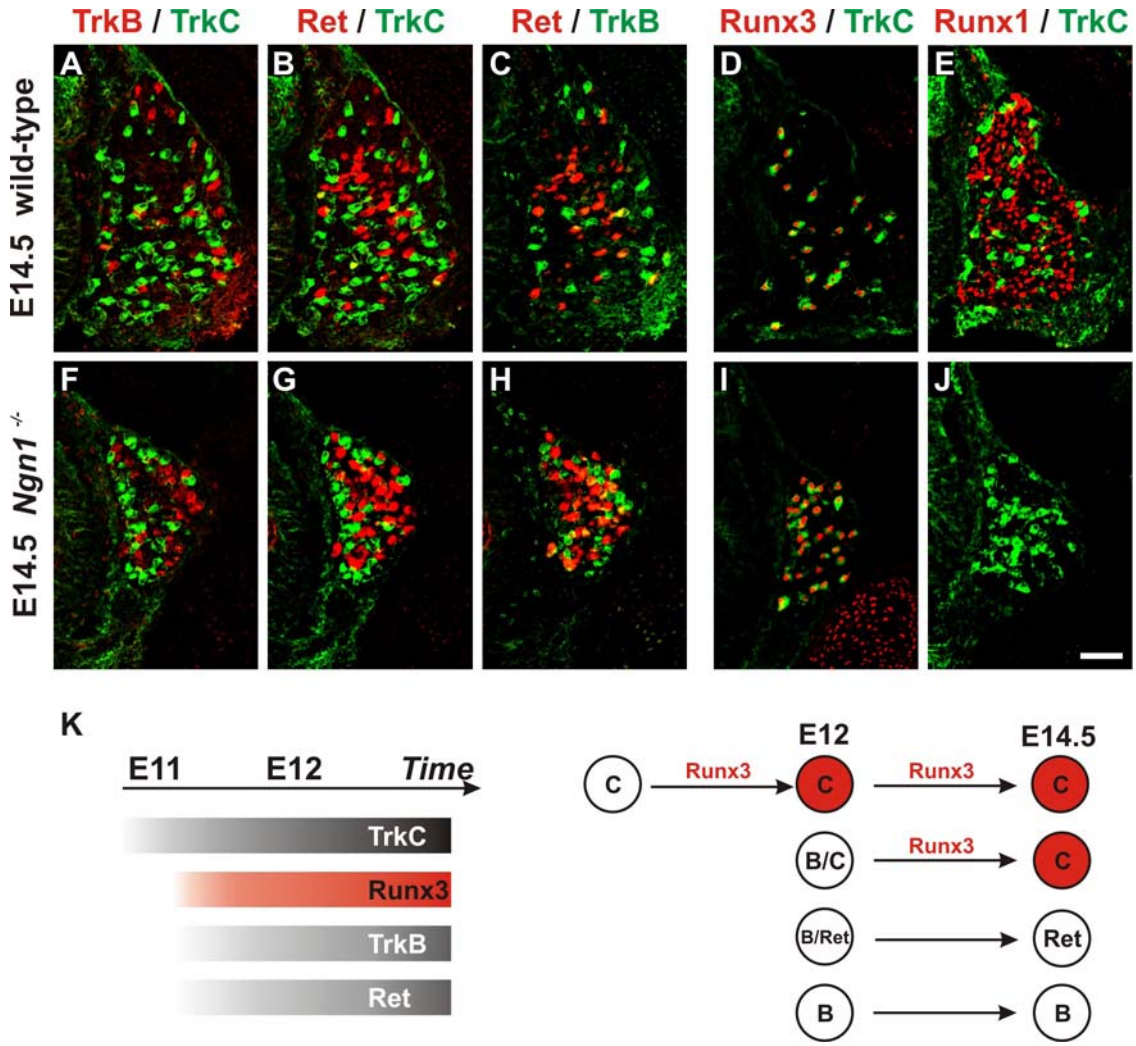


**Figure 27.** Ngn1 is not required for segregation of neurotrophic factor receptor expression in DRG sensory neurons

Immunohistochemical analysis of TrkB (red)/TrkC (green) (A, F), Ret (red)/TrkC (green) (B, G), Ret (red)/TrkB (green) (C, H), Runx3 (red)/TrkC (green) (D, I), and Runx1 (red)/TrkC (green) (E, J) expression in E14.5 lumbar DRG of wild-type (A-E) and *Ngn1*$^{-/-}$ (F-J) embryos. (K) Schematic representation of developmental profile of TrkB, TrkC, Ret and Runx3 expression. Left: onset of expression of TrkC precedes TrkB, Ret and Runx3 expression. Right: Runx3 expression coincides with appearance of a pure TrkC phenotype, but cannot be detected in hybrid neurons or TrkB+ neurons. Scale bar = 50mm.

To assess whether the generation and presence of TrkA positive sensory neurons plays a role in the segregation of hybrid TrkB+/TrkC+ and TrkB+/Ret+ DRG neurons into solitary neurotrophic factor receptor phenotypes, we analyzed *Ngn1* mutants at E14.5. We found no increase in the fraction of hybrid TrkB+/TrkC+ and TrkB+/Ret+ DRG neurons comparing wild-type and *Ngn1* mutants (Figure 27A-C, F-H). These findings

suggest that neither the generation of these neuronal phenotypes nor their segregation into distinct populations requires the presence of TrkA positive sensory neurons or *Ngn1* activity. Taken together, these data suggest that hybrid TrkB+/TrkC+ and TrkB+/Ret+ sensory neurons represent transient populations that exist at an early developmental stage, before their segregation into TrkB positive, TrkC positive or Ret positive DRG neurons (Figure 27K).

We next examined how Runx3 expression segregates with neurotrophic factor receptor expression in subsets of sensory neurons. The onset of Runx3 expression in lumbar level sensory neurons occurs at E11.5, and soon after more than 90% of TrkC positive DRG neurons coexpressed Runx3 (Figure 26A, E). In contrast, fewer than 5% of Runx3 positive sensory neurons coexpressed TrkB or Ret at this stage (Figure 26B, F). We noted that many TrkB+/TrkC+ neurons expressed low or undetectable levels of Runx3, whereas TrkC positive neurons that did not coexpress TrkB were typically associated with high levels of Runx3 expression (Figure 26A-H).

To explore further the correlation between Runx3 and TrkC expression, we analyzed mouse mutants in which the generation and/or survival of distinct classes of DRG neurons is defective. In *TrkC* mutants analyzed at E17.5, no Runx3 positive neurons were observed, consistent with the loss of proprioceptive afferents in these mice (data not shown; Klein et al., 1994; Liebl et al., 1997). Moreover, in *Ngn1* mutant embryos, Runx3 expression was maintained, and coincided with TrkC at E14.5, as in wild-type embryos (Figure 27D, I). In contrast, and despite the presence of a small number of TrkA positive DRG neurons (data not shown; Ma et al., 1999), no expression of Runx1 was detected in *Ngn1* mutants (Figure 27J) suggesting that Runx1 expression is restricted to *Ngn1*-dependent TrkA positive sensory neurons.

Taken together, these findings show that Runx3 expression coincides with a TrkC phenotype, but is not present in hybrid TrkB+/TrkC+ and TrkB+/Ret+ neurons. The exclusion of Runx3 expression from these hybrid neuronal populations raises the possibility that its status of expression has a role in the segregation of neurotrophic factor receptor phenotypes in sensory neurons.

## ALTERING THE STATUS OF RUNX3 IN DEVELOPING DRG SENSORY NEURONS

To determine whether the status of Runx3 expression defines the neurotrophic factor receptor phenotype of early born sensory neurons, we compared the consequences of ectopic expression of Runx3 and of elimination of Runx3 function from sensory neurons.

To express Runx3 ectopically in all sensory neurons soon after their exit from the cell cycle, we used homologous recombination in ES cells to integrate into the *tau* locus a cassette suitable for Cre recombinase-mediated activation of *Runx3* and *lacZ* expression from a bi-cistronic message (Figure 28A, B; Tucker et al., 2001; Hippenmeyer et al., 2005). To achieve expression of Runx3 in all sensory neurons from early postmitotic stages, $Tau^{Runx3}$ mice were crossed to a strain of mice with an integration of *Cre* recombinase into the *Isl1* locus (Srinivas et al., 2001). In $Tau^{Runx3/+}$ $Isl^{Cre}$ embryos analyzed at E17.5, efficient expression of Runx3 and LacZ was observed in >95% of sensory neurons (Figure 28F-H). To determine whether the actions of Runx3 are cell autonomous, we crossed $Tau^{Runx3}$ mice with $Hb9^{Cre}$ mice in which activation of the $Tau^{Runx3}$ allele is achieved only in a subset of DRG neurons (Tucker et al., 2001; Hippenmeyer et al., 2005). To eliminate *Runx3* function, we used constitutive *Runx3* mutant mice generated in the Littman laboratory by deleting the exon encoding the C-terminal half of the DNA binding runt domain from the *Runx3* locus. In these mice, expression of a non-functional fragment of Runx3 protein ($Runx3^{trunc}$) can still be detected, and thus provides a molecular tag with which to trace prospective *Runx3* sensory neurons in $Runx3^{-/-}$ embryos.

## ALTERED RUNX3 EXPRESSION INFLUENCES THE SEGREGATION OF TRKB AND TRKC EXPRESSION

We first examined the consequences of ectopic expression of Runx3 on the developmental profile of TrkB and TrkC expression. A prediction of the hypothesis that Runx3 plays a role in the acquisition of the solitary TrkC phenotype is that ectopic expression of Runx3 will block the potential for TrkB expression in DRG neurons.

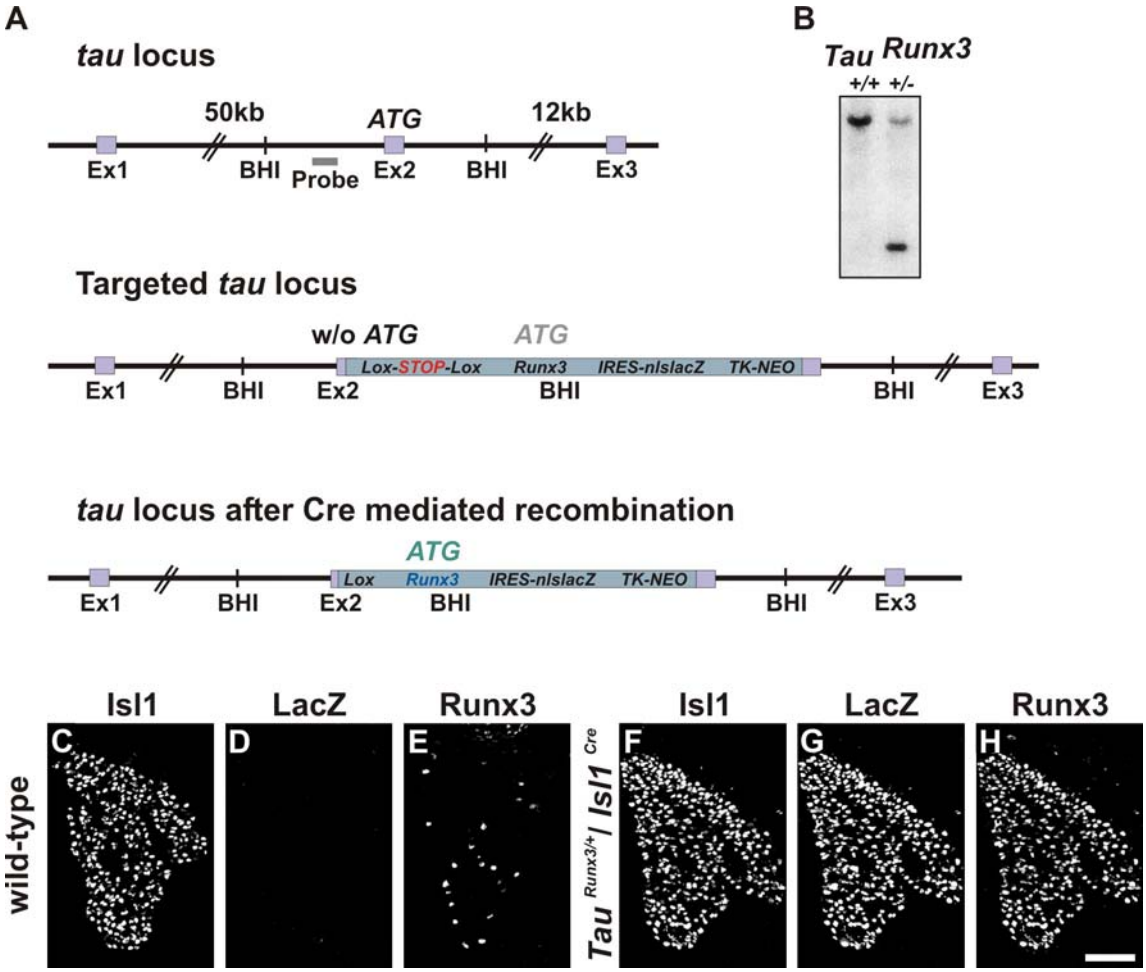


**Figure 28.** Ectopic expression of Runx3 in DRG neurons

**(A)** (Top panel) Organization of the *tau* genomic locus in the region targeted by homologous recombination (Tucker et al., 2001; Hippenmeyer et al., 2005). 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 cassette (green) into exon 2 with coincident elimination the endogenous *tau* start codon. In the absence of Cre recombinase, a transcriptional stop sequence flanked by *lox*P sites inhibits expression of the transgene from its start codon (*ATG* in grey). The integrated targeting cassette allows for conditional expression of *Runx3* and *nls-lacZ* upon Cre recombinase-mediated activation (*ATG* in green). **(B)** Southern blot analysis of $Tau^{Runx3/+}$ genomic DNA of F1 offspring to detect the mutant allele. **(C-H)** Expression of Isl1 (C, F), LacZ (D, G) and Runx3 (E, H) in E17.5 lumbar DRG neurons of wild-type (C-E) and $Tau^{Runx3/+}$ $Isl1^{Cre}$ (F-H) embryos. Scale bar: = 88 μm.

We compared the acquisition of TrkB expression in E12 sensory neurons in $Tau^{Runx3/+}$ $Isl^{Cre}$ and wild-type embryos. TrkB expression was completely abolished in DRG sensory neurons in $Tau^{Runx3/+}$ $Isl^{Cre}$ embryos (Figure 29D, E). Thus, Runx3 is capable of suppressing TrkB expression in sensory neurons. In $Tau^{Runx3/+}$ $Isl^{Cre}$ embryos, the total number of TrkC positive sensory neurons analyzed at E13.5 was increased by only 20%

(see below), suggesting that prospective TrkB neurons do not assume a TrkC phenotype. Moreover, in *Tau*$^{Runx3/+}$ *Hb9*$^{Cre}$ embryos, in which productive Cre-mediated recombination events occur only in very few sensory neurons at brachial levels, we detected mutually exclusive expression of LacZ and TrkB in sensory neurons throughout all rostro-caudal levels (Figure 30C, G). In control experiments, activation of EGFP and LacZ expression from a bi-cistronic message using the same genetic strategy (*Tau*$^{mGFP\text{-}INLA/+}$ *Hb9*$^{Cre}$; Hippenmeyer et al., 2005) resulted in the presence of TrkB+/LacZ+ neurons at both brachial and lumbar levels of the spinal cord (Figure 30A, E). Thus, Runx3 represses TrkB expression in sensory neurons, and appears to do so in a cell autonomous manner.

We next examined whether the loss of *Runx3* function also influences the segregation of TrkB and TrkC expression in sensory neurons. We compared the profile of TrkB expression in sensory neurons in *Runx3*$^{-/-}$ and wild-type embryos at E12. We detected a 2.1-fold increase in the total number of TrkB positive neurons in *Runx3* mutants (Figure 29G, H). In addition, over 95% of all remaining TrkC positive neurons in *Runx3* mutant embryos coexpressed TrkB (Figure 29H), suggesting that ectopic expression of TrkB in TrkC positive neurons underlies the increased number of TrkB positive sensory neurons in *Runx3* mutants. Consistent with this interpretation, many TrkB positive sensory neurons in *Runx3*$^{-/-}$ embryos were marked by coexpression of Runx3$^{trunc}$ (Figure 29G), whereas in wild-type sensory neurons, Runx3 and TrkB expression rarely, if ever, overlapped (Figure 26B, F; Figure 29A).

Together, the complementary outcomes of these ectopic expression and loss of function experiments suggest that Runx3 normally promotes the transition from a transient and hybrid TrkC+/TrkB+ phenotype to a solitary TrkC positive phenotype, by repressing the potential for TrkB expression within prospective proprioceptors. These data also reveal the capacity of Runx3 to repress TrkB expression in prospective TrkB positive sensory neurons.

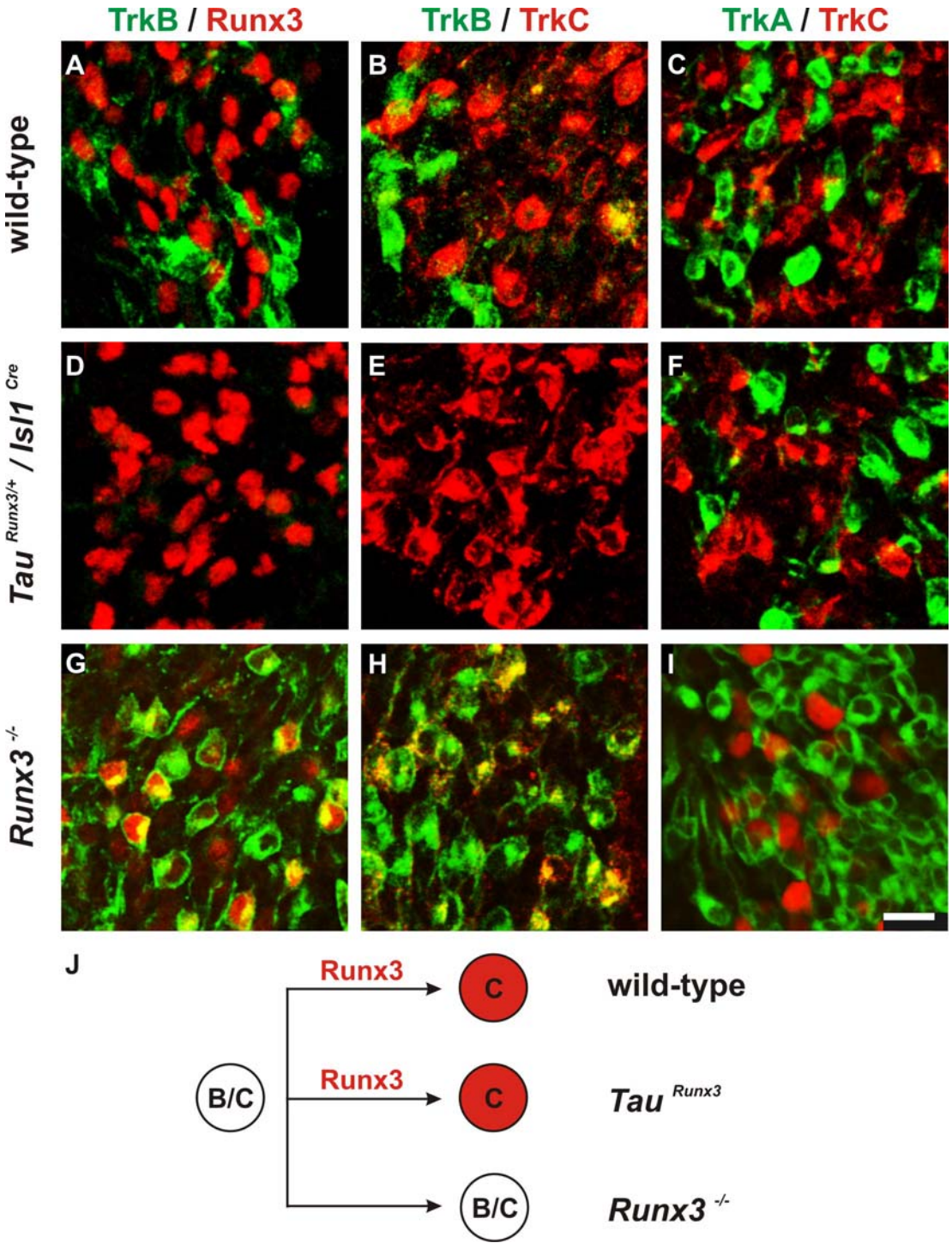


**Figure 29.** Runx3 promotes transition from TrkB/TrkC to TrkC neurons

Immunohistochemical analysis of TrkB (green)/Runx3 (red) (A, D, G), TrkB (green)/TrkC (red) (B, E, H) and TrkA (green)/TrkC (red) (C, F, I) expression on E12 lumbar DRG of wild-type (A-C), *Tau*$^{Runx3/+}$ *Isl1*$^{Cre}$ (D-F) and Runx3$^{-/-}$(G-I) embryos. (J) Summary diagram of the effects of gain- and loss-of-function experiments on hybrid TrkB+/TrkC+ sensory neurons. Ectopic Runx3 expression blocks the potential for TrkB expression in DRG neurons whereas Runx3 mutant prospective TrkC+ proprioceptors aberrantly express TrkB. Scale bar = 15 μm.

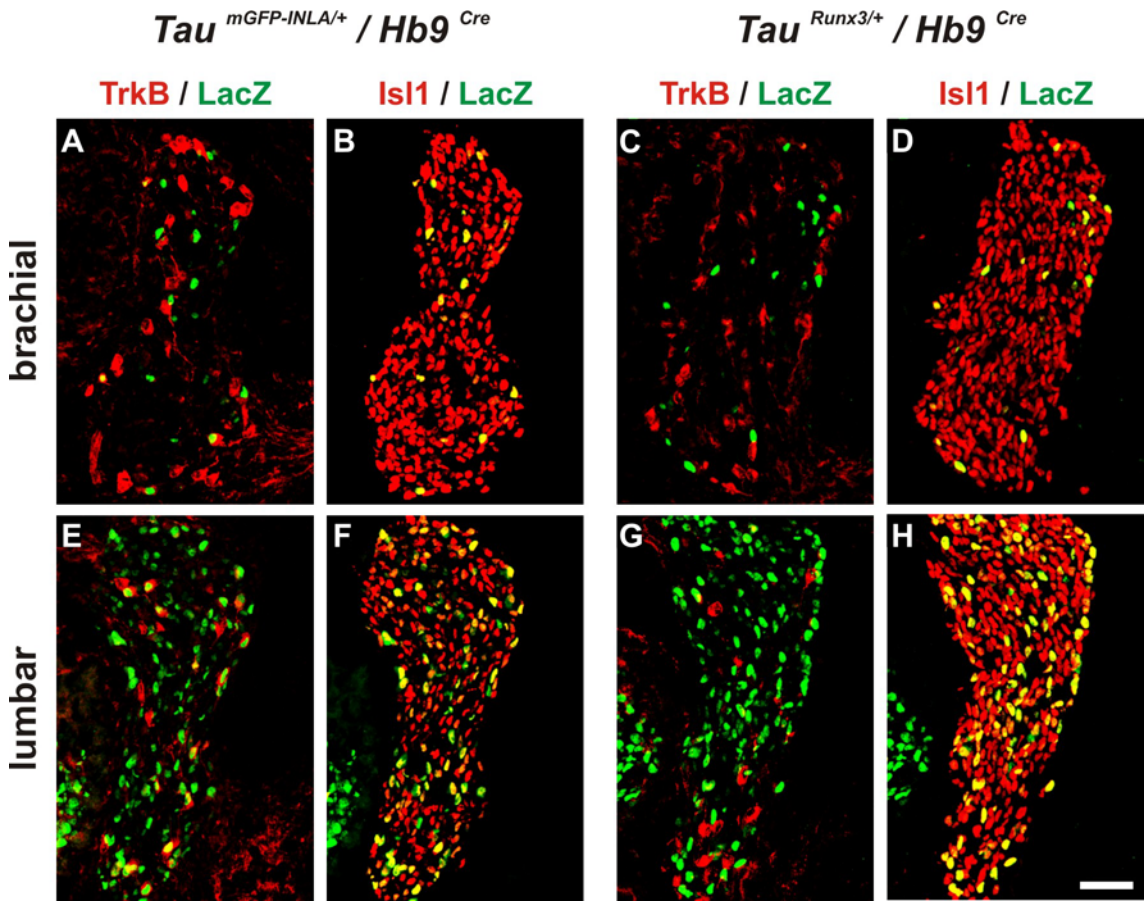


**Figure 30.** Runx3 acts cell autonomously to repress TrkB in DRG neurons

Triple labeling experiment to detect TrkB (A, C, E, G: red), LacZ (A-H: green) and Isl1 (B, D, F, H: red) expression in E13.5 brachial (A-D) or lumbar (E-H) DRG of *Tau*$^{mGFP\text{-}INLA/+}$ *Hb9*$^{Cre}$ (A, B, E, F) or *Tau*$^{Runx3/+}$ *Hb9*$^{Cre}$ (C, D, G, H) embryos. Scale bar = 45 μm.

## ALTERED RUNX3 EXPRESSION INFLUENCES THE SEGREGATION OF RET AND TRKB EXPRESSION

We next examined how the status of Runx3 expression influences the normal developmental segregation of TrkB from Ret expression. In *Tau*$^{Runx3/+}$ *Isl*$^{Cre}$ embryos, TrkB expression could no longer be detected in Ret positive neurons, indicating that Runx3 effectively represses TrkB expression within TrkB+/Ret+ neurons (Figure 31D). In addition, the association of Ret and Trk expression was dramatically altered. Whereas Ret positive sensory neurons are invariably associated with TrkB expression in wild-type embryos at E12 (Figure 26J-L; Figure 31A), in *Tau*$^{Runx3/+}$ *Isl*$^{Cre}$ embryos, 80% of all Ret positive neurons coexpressed TrkC (Figure 31E). And as described above, TrkB

expression was eliminated (Figure 31D). In addition, we observed a 1.2-fold increase in the total number of TrkC positive sensory neurons in $Tau^{Runx3/+}$ $Isl^{Cre}$ embryos analyzed at E13.5 (wild-type: 323 ± 12; $Tau^{Runx3/+}$ $Isl^{Cre}$: 374 ± 31; p = 0.013). In contrast, coexpression between Ret and TrkA was not detected at these early stages (Figure 31F).

Together, these findings imply that within the cellular context of Ret positive sensory neurons, Runx3 acts coordinately to extinguish TrkB and activate TrkC expression. Nevertheless, the number of differentiated proprioceptive afferents assessed at E17.5 by expression of the calcium binding protein parvalbumin (Arber et al., 2000), was not significantly increased by ectopic expression of Runx3 (data not shown), suggesting that Runx3 activity is not sufficient to support emergence of a fully differentiated proprioceptor state in Ret positive DRG neurons.

What then is the consequence of loss of Runx3 function on the early Ret positive sensory neuron population? We detected no change in the number of TrkB+/Ret+ neurons and no TrkC+/Ret+ or TrkA+/Ret+ neurons in *Runx3* mutant embryos (Figure 31G-I). Thus, Runx3 activity does not promote the progression from a transient TrkB+/Ret+ to a Ret positive sensory neuron phenotype. Finally, we determined whether Runx3 activity is able to promote TrkC expression in TrkA positive sensory neurons. As in wild-type embryos, few if any hybrid TrkA+/TrkC+ DRG sensory neurons were detected under conditions of ectopic *Runx3* overexpression or in *Runx3* mutants (Figure 29C, F, I). This finding effectively eliminates the possibility that the exclusion of TrkA expression from TrkC positive proprioceptive sensory neurons is mediated by Runx3. Moreover, these findings reveal that ectopic Runx3 is unable to induce TrkC expression within the context of TrkA positive sensory neurons.

Together, our findings argue that during normal DRG sensory neuron development Runx3 exerts a pervasive repressive influence on the acquisition of the TrkB phenotype, yet appears able to direct a TrkC phenotype only within the limited cellular context of Ret positive sensory neurons.

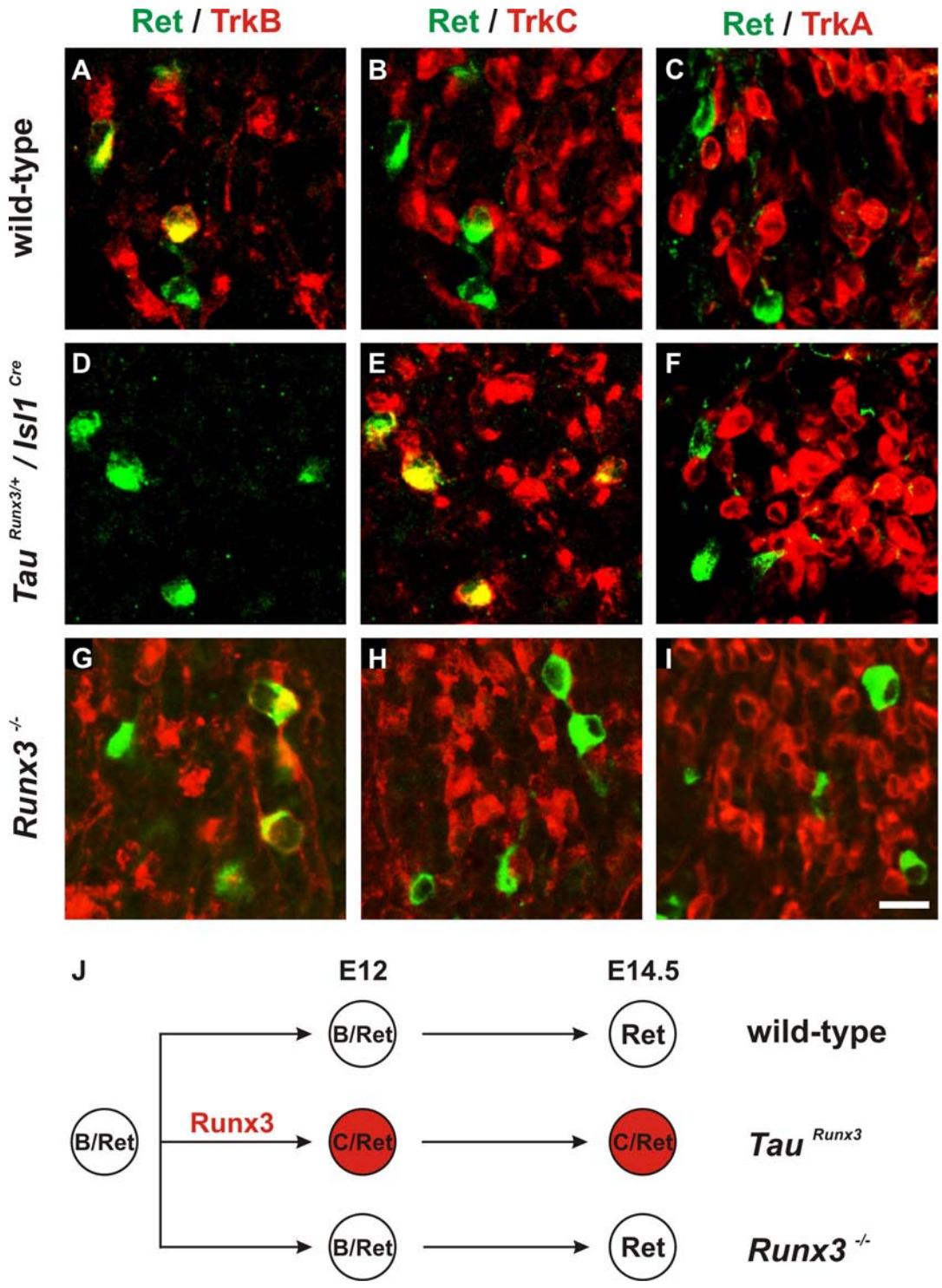


**Figure 31.** Runx3 induces TrkC in Ret sensory neurons

Immunohistochemical analysis of Ret (A-I: green) and TrkB (A, D, G: red) or TrkC (B, E, H: red) or TrkA (C, F, I: red) expression in E12 lumbar DRG of wild-type (A-C), *Tau*$^{Runx3/+}$ *Isl1*$^{Cre}$ (D-F) and *Runx3*$^{-/-}$ (G-I) embryos. (J) Summary diagram of the effects of gain- and loss-of-function experiments on Ret+/TrkB+ sensory neurons. Loss of Runx3 expression does not affect Trk expression in Ret+ sensory neurons but ectopic Runx3 expression induces the potential for TrkC expression in these neurons. Scale bar = 15μm.

# DISCUSSION

Neuronal differentiation involves the acquisition of many specialized molecular properties that are essential for later neuronal function. Few aspects of the differentiated neuronal phenotype have a greater functional impact than the expression of neurotrophic factor receptors. At different developmental stages, the precision of neurotrophic factor receptor expression determines the survival of neurons (Bibel and Barde, 2000; Huang and Reichardt, 2003), axonal branching patterns (Bibel and Barde, 2000; Hippenmeyer et al., 2004), and the strength of synaptic connections (Schuman, 1999; Arvanian et al., 2003). Yet the mechanisms that establish the selectivity of neurotrophic factor receptor expression by specific populations of vertebrate neurons remain obscure. In this study we have examined how different functional subpopulations of primary sensory neurons in the DRG acquire selective profiles of neurotrophic factor receptor expression that ensure the maturation of distinct proprioceptor and mechanoreceptor subtypes. We provide evidence that the runt domain transcription factor Runx3 has a key role in establishing the proprioceptor phenotype, by promoting the transition from an intermediate TrkB/TrkC hybrid phenotype to a selective TrkC phenotype.

## RUNX3 ACTIVITY AND THE EMERGENCE OF A PROPRIOCEPTOR PHENOTYPE

The phenotypic changes observed after gain and loss of Runx3 function in DRG neurons suggest a model in which newly generated sensory neurons can be divided into two broad populations with distinct developmental potentials. One neuronal class, capable of differentiating into mechanoreceptive or proprioceptive sensory neurons initially possesses the potential to express TrkB. But this population rapidly fragments into three distinct subclasses, defined by expression of TrkC, TrkB or TrkB and Ret. A second neuronal class, destined to differentiate into nociceptors, initially expresses TrkA, but later fragments into distinct populations that express either TrkA or Ret (Molliver et al., 1997). Runx3 appears to have a key role within the first class of sensory neurons, driving the progression of neurons from an intermediate TrkB/TrkC hybrid phenotype into a TrkC proprioceptor phenotype, by repressing TrkB and promoting TrkC expression.

Several lines of evidence support a model in which the emergence of a pure TrkC proprioceptive sensory neuron phenotype depends on Runx3 activity. First, sensory neurons that display a hybrid TrkB/TrkC phenotype can normally be detected at early developmental stages, and this hybrid Trk phenotype correlates with absence of Runx3 expression. In contrast, prospective proprioceptors that exhibit a pure TrkC character express Runx3. Further support for the existence of a transient TrkB/TrkC hybrid sensory phenotype has been provided in recent genetic tracing experiments which show that more than 70% of trigeminal ganglion sensory neurons with a TrkB character coexpress TrkC at early developmental stages (Funfschilling et al., 2004). In addition, ectopic expression of Runx3 in sensory neurons efficiently suppresses the potential for TrkB expression and promotes TrkC expression, albeit within a limited neuronal context. Finally, in *Runx3* mutant mice analyzed at E12.5, all remaining TrkC positive sensory neurons coexpress TrkB.

## CONSTRAINTS ON RUNX3 EXPRESSION AND FUNCTION WITHIN DRG NEURONS

Our findings imply that the exclusion of Runx3 from TrkB positive and Ret positive sensory neurons is essential for the consolidation of mechanoreceptive phenotypes. Amongst sensory neurons with a TrkB, TrkC or Ret character analyzed at E11.5 to E12, the only neurons that lack TrkB expression are those that express Runx3, suggesting that the competence to express TrkB is present broadly in early sensory neurons and that one function of Runx3 is to counteract the establishment of a TrkB character in prospective proprioceptors. The onset of TrkB and Runx3 expression is virtually coincident in DRG neurons, occurring somewhat after that of TrkC, providing a potential explanation of why so few neurons coexpressing TrkB and TrkC are detected. Presumably only those neurons that initiate expression of TrkB before that of Runx3 gain the chance to coexpress both Trk proteins. Nevertheless, our findings do not rule out the possibility that the emergence of neurons with a TrkB phenotype requires an activity complementary to that of Runx3, which functions to control the extinction of a TrkC phenotype within hybrid TrkB/TrkC sensory neurons. In this scenario, the finding that Runx3 can suppress the emergence of a

TrkB phenotype in neurons that are not fated to become proprioceptors suggests that Runx3 is dominant over any factor involved in promoting the acquisition of the TrkB phenotype.

We find that widespread misexpression of Runx3 in DRG neurons promotes the emergence of an ectopic TrkC phenotype in only a small subset of neurons. No ectopic induction of TrkC was detected in TrkA positive sensory neurons, but most early emerging Ret positive sensory neurons consistently coexpressed TrkC upon misexpression of Runx3. The extinction of TrkB after Runx3 expression under these experimental conditions prevents direct assessment of whether the emergence of a TrkC phenotype is also promoted within neurons with prospective TrkB character. The restricted cellular context in which Runx3 drives ectopic TrkC expression poses the question of the nature of the constraints on Runx3 activity in different classes of sensory neurons. One intriguing possibility is that the *Ngn1*-dependent sensory neurons, which constitute the majority of all TrkA positive neurons, are resistant to Runx3 activity, at least with respect to initiation of TrkC expression. Consistent with this possibility, recent studies have indicated that ectopic *TrkC* expression from the *TrkA* locus induces only a small fraction of all nociceptive sensory neurons to convert to a proprioceptive afferent phenotype (Moqrich et al., 2004), a further indication of a restriction in cellular context in which conversion of sensory neuronal phenotype can be achieved.

Together, these findings suggest the existence of a major sensory neuron subtype barrier that defies transitions between neurotrophic factor receptor selection and expression. We have detected no TrkA/TrkC or TrkA/TrkB hybrid characters during the period of generation of the majority of TrkA positive sensory neurons, suggesting that TrkA emerges independently of TrkB/TrkC character. Furthermore, many trigeminal ganglion neurons with TrkB phenotype coexpress TrkC at one point during their development, whereas very few TrkA positive neurons emerge from a transient TrkC positive population (Funfschilling et al., 2004). The coexpression of TrkC with Runx3, and of TrkA with Runx1 indicates that the segregation in expression of Runx3 and Runx1 also conforms to this sensory neuron subtype barrier.

The emerging role for Runx3 in selection of proprioceptor phenotype raises the issue of whether Runx1 fulfills a role within the TrkA positive nociceptor population similar to that of Runx3 within the TrkB/TrkC neuronal cohort. Within the subpopulation of nociceptive sensory neurons with TrkA character, two major classes of sensory neurons can be distinguished on the basis of their distinct profiles of neurotrophic factor receptor expression. Non-peptidergic nociceptive neurons undergo a switch in neurotrophic factor receptor expression from TrkA to Ret at late embryonic stages (Molliver et al., 1997), whereas other nociceptive sensory neurons maintain TrkA and coexpress neuropeptides such as calcitonin gene related peptide (CGRP; Scott, 1992). We have found that ectopic expression of Runx1 within TrkA positive sensory neurons represses CGRP, a marker of a neuronal subpopulation normally largely excluded from Runx1 positive DRG neurons (Kramer and Arber, unpublished observation). Conversely, a significant increase in the number of CGRP positive nociceptive neurons is observed in *Runx1* mutant DRG (Ma et al., personal communication). Together, these findings suggest that Runx3 and Runx1 may act in a similar manner within the two major subtypes of sensory neurons, to further subdivide specific neuronal populations.

## THE CONSEQUENCES OF HYBRID TRK PHENOTYPES IN DRG NEURONS

How does the emergence of a hybrid TrkB/TrkC phenotype affect the later differentiation of sensory neurons? We observed a lack of parvalbumin expression and no increase in TrkB positive sensory neurons at late embryonic stages in our *Runx3* mutant mice, suggesting that hybrid TrkB+/TrkC+ cells are eliminated. In support of this view, proprioceptive afferents in *Runx3* mutant mice have previously been shown to undergo cell death (Levanon et al., 2002). We also assessed whether ectopic Runx3 expression promotes the emergence and differentiation of an additional complement of mature proprioceptive neurons within the DRG. But we found no increase in the number of proprioceptive DRG neurons at late embryonic stages, at least as assessed by expression of the proprioceptor marker parvalbumin (Schuman, 1999; Moqrich et al., 2004). Taken together, therefore, our findings suggest that Runx3 expression within proprioceptors is

necessary for acquisition of late proprioceptive phenotypes, but is not sufficient to support a complete phenotypic conversion outside the cellular context of proprioceptors.

## CONSERVED ROLES FOR RUNX PROTEINS IN SPECIFICATION OF CELLULAR PHENOTYPES

The involvement of Runx3 in the establishment of a proprioceptive neuronal phenotype has intriguing parallels with other studies of the function of Runx transcription factors. Within the nervous system, runt domain transcription factors have been studied intensively in the context of their role in the differentiation of distinct photoreceptor subtypes in the Drosophila eye. Amongst all photoreceptors, Runt is selectively expressed in R7 and R8 photoreceptors, but excluded from R1-R6 and functional evidence suggest that Runt controls important aspects of R7/R8 photoreceptor differentiation (Kaminker et al., 2002; Tayler and Garrity, 2003). Moreover, Runx1 has been shown to have a role in controlling proliferation of selected progenitor cells in the brain and proposed to act by repression of the cell cycle inhibitor p21 (Therilaut et al., 2005). Thus, Runx proteins may have conserved roles in controlling neuronal phenotype.

In addition, Runx proteins exert a pivotal role in the control of thymocyte differentiation (Taniuchi et al., 2002b). As thymocytes mature, they progress from a transient $CD4^+CD8^+$ cell type to give rise to either $CD4^-CD8^+$ cytotoxic or $CD4^+CD8^-$ helper T cells (Taniuchi et al., 2002b). In the absence of Runx3 function, transcriptional silencing of CD4 is impaired, resulting in the development of $CD4^+CD8^+$ cytotoxic T cells that have additional functional deficits. However, development of $CD4^+CD8^-$ helper thymocytes, which do not express Runx3, is not affected by this mutation. Together, these findings suggest a pervasive role for Runx proteins in the emergence of mature cellular phenotypes and functions in both the nervous and immune system.

# METHODS

## GENERATION OF MICE AND MOUSE GENETICS

Generation of *Tau*$^{Runx3}$ mice: a *lox-STOP-lox-Runx3-IRES-NLS-LacZ-pA* targeting cassette was integrated into exon 2 of the *tau* genomic locus (the endogenous start ATG was removed in the targeting vectors; details available upon request). ES cell recombinants were screened by Southern blot analysis using a probe in the 5' region as described previously (Tucker et al., 2001; Hippenemyer et al., 2005). Frequency of recombination in 129/Ola ES cells was around 1:3. Recombinant ES cell clones were aggregated with morula-stage embryos to generate chimeric founder mice that transmitted the mutant allele.

Dan Littman generously provided *Runx3* mutant mice prior to publication. In these mice, a conditional allele of *Runx3* was generated by flanking exon 4 of the *Runx3* locus by *lox*P sites. These mice were crossed with a mouse strain expressing Cre recombinase ubiquitously under the control of the TK promoter (Bai et al., 2002) to achieve germ-line transmission of a *Runx3* mutant allele (details on the generation of these mice will appear in a separate study).

In all experiments performed in this study, animals were of mixed genetic background (129/Ola and C57Bl6). *Isl1*$^{Cre}$ (Srinivas et al., 2001), *Ngn1*$^{+/-}$ (Ma et al., 1999), *TrkC*$^{+/-}$ (Liebl et al., 1997), *TrkA*$^{+/-}$ (Moqrich et al., 2004) and *Tau*$^{mGFP-INLA}$ (Hippenmeyer et al., 2005) mouse strains have been described previously. To achieve expression of Runx3 only in a subset of DRG neurons, we used *Hb9*$^{Cre}$ mice (Yang et al., 2001; Hippenmeyer et al., 2005). Due to the early transient expression of *Hb9* at neural plate stages in a rostro-caudally increasing gradient, this strategy leads to a gradual increase in the frequency of Cre-mediated recombination events resulting in very few recombined neurons at brachial levels to many neurons in lumbar level DRG (Hippenmeyer et al., 2005). Timed pregnancies were set up to generate embryos of different developmental stages.

## IMMUNOHISTOCHEMISTRY AND QUANTIFICATION

Antibodies used in this study were: rabbit anti-Runx3 (generated against a 6xHis tagged carboxy-terminal fusion protein of Runx3; amino acids 187-415), rabbit anti-Runx3 (generated against a mouse Runx3 peptide: AQATAGPGGRTRPEVRS), rabbit anti-Runx1 (generated against a mouse Runx1 peptide: GRASGMTSLSAELSSRL), rabbit anti-TrkA and -p75 (gift from L.F. Reichardt), rabbit anti-Ret (IBL), rabbit anti-parvalbumin (SWANT), goat anti-Ret (RDI), goat anti-LacZ (Arber et al., 2000), goat anti-TrkC (gift from L.F. Reichardt), chick anti-TrkB (gift from L.F. Reichardt), guinea pig anti-Isl1 (Arber et al., 2000). Cryostat sections were processed for immunohistochemistry as described (Arber et al., 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes). Images were collected on an Olympus confocal microscope. For quantitative analysis of hybrid neurotrophic factor receptor expressing neurons, the incidence of coexpression was determined in L3 and L4 DRG in at least three independent experiments. For quantification of Ret and TrkC positive DRG neurons, Ret+/Isl1+ or TrkC+/Isl1+ neurons were counted at 20 μm intervals throughout the entire L3 DRG. For statistical analysis, counts from at least 6 L3 DRG of each genotype were compared using an unpaired Student's *t*-test.

# CHAPTER 8

# INTERACTION OF RUNX AND ETS TRANSCRIPTION FACTORS

# INTRODUCTION

One important issue that has not yet been addressed in much detail is whether and how Runx3 is involved in controlling later aspects of proprioceptive sensory neuron specification such as the establishment of correct sensory-motor circuitry. Since proprioceptive sensory neurons are eventually lost in *Runx3*$^{-/-}$ embryos, as was published in the first study addressing the role of Runx3 in sensory neuron differentiation (Levanon et al., 2002), determination of the potential later functions of Runx3 would require analysis of conditional *Runx3* mutants, in which *Runx3* function is eliminated only at later time points of development. However, in a second report using independently generated *Runx3* knockout mice, it was found that *Runx3* deficient proprioceptive neurons survive and express proprioceptive neuronal markers such as TrkC, parvalbumin and Er81 (Inoue et al., 2002). Moreover, absence of *Runx3* was associated with severe axonal projection defects of proprioceptive neurons, both centrally as well as peripherally leading to a highly ataxic phenotype of *Runx3* deficient mice. While the underlying mechanisms giving rise to the observed different phenotypes of loss of *Runx3* function remain obscure, perhaps reflecting in part the impact of different genetic backgrounds of the two mouse strains analyzed, these conflicting results indicate that Runx3 is likely to control additional important steps in proprioceptive sensory neuron specification.

Interestingly, it has previously been reported that Runx and ETS transcription factors can functionally interact *in vitro* by mutually enhancing their DNA binding and transactivation capacities through reciprocal relief from intramolecular autoinhibition (Wotton et al., 1994; Erman et al., 1998; Sato et al., 1998; Kim et al., 1999b; Mao et al., 1999; Goetz et al., 2000; Gu et al., 2000). The majority of these earlier interaction studies focused on the cooperation of the Runx family member Runx1 with the ETS transcription factor Ets1 at the T cell receptor (TCR) α and β enhancers, which contain several Runx and ETS DNA binding site in close proximity and constitute well characterized *in vivo* targets of Runx and ETS transcription factors (Ito, 1999).

Given these findings and the fact that Runx3 is coexpressed with the ETS transcription factor Er81 in proprioceptive DRG sensory neurons, it is conceivable that also Runx3 and Er81 might functionally interact to control late steps in proprioceptive sensory neuron

specification. By performing co-immunoprecipitation experiments to test for protein-protein interaction we could demonstrate that Runx3 and Er81 interact *in vitro.* In addition, we found that Runx3 and Er81 interact genetically, as *Er81* is required for maintaining Runx3 expression in a subset of proprioceptive DRG neurons *in vivo*. Finally, we analyzed whether ectopic expression of Runx3 was able to rescue the *Er81* knockout phenotype, in which proprioceptive afferents fail to project towards their normal central target area in the ventral horn of the spinal cord and instead terminate prematurely in the intermediate region of the spinal cord.

## RESULTS AND DISCUSSION

### RUNX AND ETS TRANSCRIPTION FACTORS INTERACT *IN VITRO*

To test whether Runx3 and Er81 interact we first turned to an *in vitro* system by performing co-immunoprecipitation assays using transiently cotransfected COS-7 cells expressing Runx3 as well as N-terminally Myc-tagged Er81 (Figure 32A). In a separate positive control experiment we also transiently cotransfected COS-7 cells with expression plasmids for Runx1 and N-terminally Myc-tagged Ets1, whose protein-protein interaction has been extensively studied in the past (Kim et al., 1999b; Goetz et al., 2000; Gu et al., 2000). Using mouse monoclonal antibodies directed against the N-terminal Myc-tag of the ETS transcription factors we were able to co-immunoprecipitate not only Runx1 from cell lysates containing Runx1 and Myc-Ets1, but also Runx3 from Runx3 and Myc-Er81 coexpressing COS-7 cells (Figure 32B). In control experiments with mouse monoclonal antibodies directed against a Flag-tag neither Runx1 nor Runx3 were co-immunoprecipitated (Figure 32B), demonstrating that the observed interaction between Runx and ETS transcription factors is specific.

In addition, by performing similar co-immunoprecipitation assays with COS-7 cells transiently expressing Runx1 and Myc-tagged Pea3 we were able to demonstrate interaction of these two transcription factors *in vitro* (data not shown).

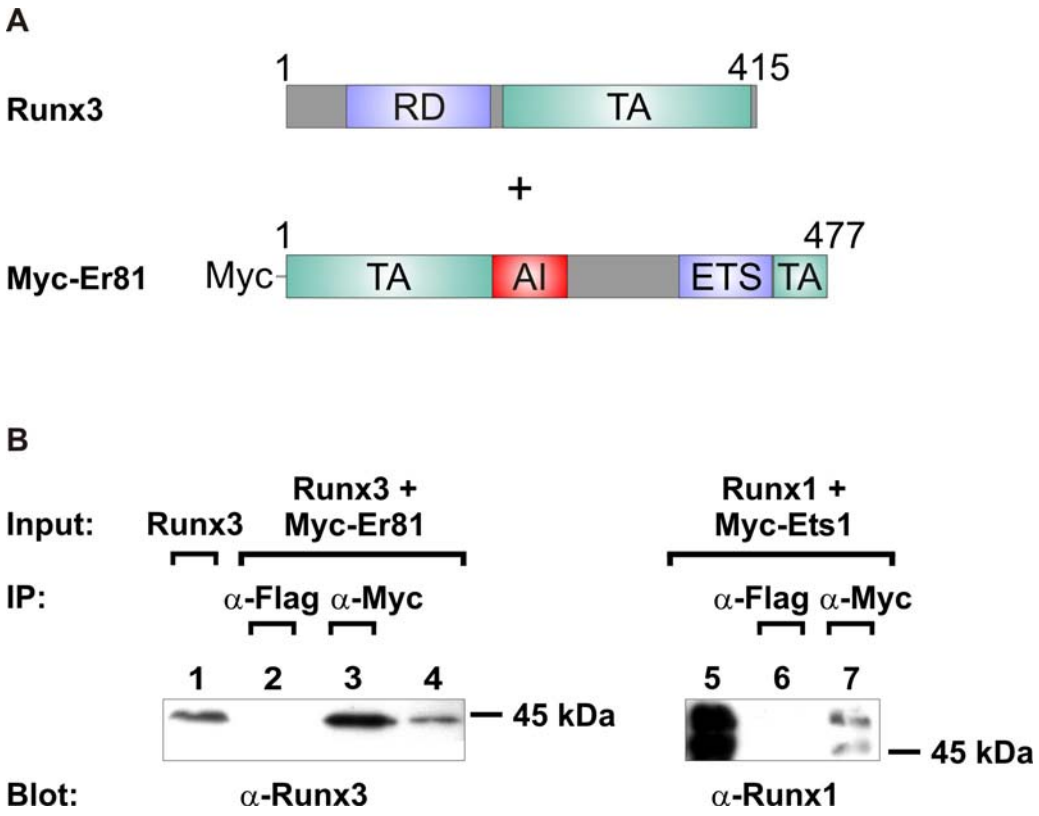


**Figure 32.** Runx and ETS transcription factors interact *in vitro*

**(A)** Domain structure of Runx3 and Myc-tagged Er81 transcription factors as expressed from expression plasmids used in co-immunoprecipitation assays. Abbreviations: AI = auto-inhibitory domain; ETS = DNA binding domain; RD = DNA binding runt domain; TA = transactivation domain. **(B)** COS-7 cells were transiently transfected with expression plasmids for Runx3 alone (lane 1) or Runx3 and Myc-Er81 (lane 2-4) or as positive control experiment for Runx1 and Myc-Ets1 (lane 5 - 7). Runx3 containing protein complexes were co-precipitated with a monoclonal anti-Myc (lane 3) but not with a monoclonal anti-Flag antibody (lane 2). As positive control experiment, Runx1 containing complexes were co-precipitated with a monoclonal anti-Myc (lane 7) but not with a monoclonal anti-Flag antibody (lane 6). The untreated cell extracts (lane 1, 4, 5) and IP extracts (lane 2, 3, 6, 7) were subjected to Western blotting with anti-Runx3 antibodies (lane 1 - 4) or anti-Runx1 antibodies (lane 5 to 7).

This observation is intriguing as in contrast to Runx3, which is exclusively expressed by sensory neurons, Runx1 is also expressed in other components of the monosynaptic stretch reflex circuit, namely in a defined set of cervical LMCm motor neurons (Figure 33; data not shown). Interestingly, these motor neurons overlap towards the caudal end of the Runx1 expression domain with motor neurons expressing Pea3, which innervate the *cutaneous maximus* and *latissimus dorsi* muscles in the periphery (Haase et al., 2002; Livet et al., 2002).

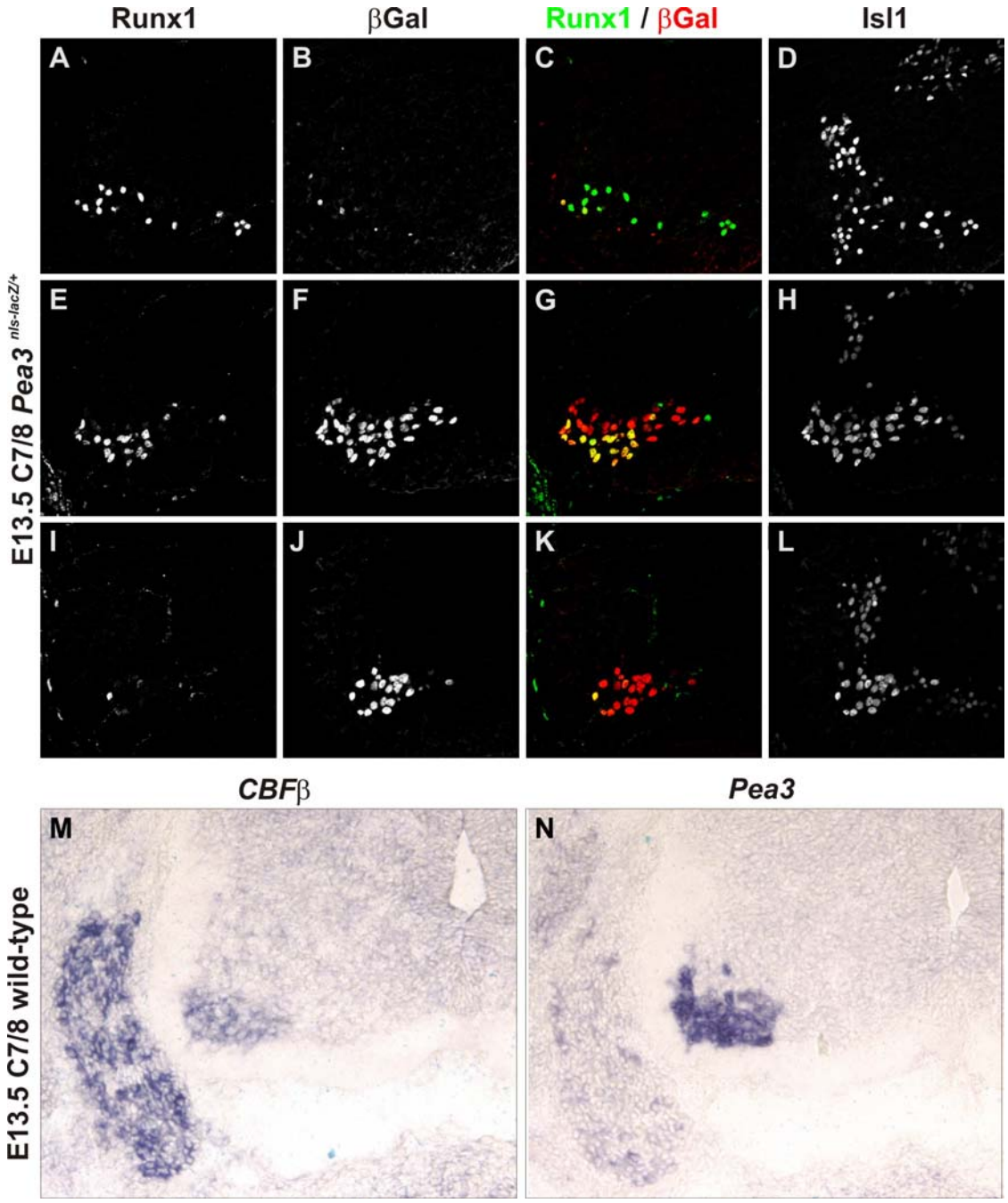


**Figure 33.** Runx1 is partly coexpressed with Pea3 in cervical LMCm motor neurons

Analysis of E13.5 cervical level 7/8 (C7/8) motor neurons in *Pea3*$^{nls\text{-}lacZ/+}$ embryos by immunohistochemistry (A-L) or in wild-type embryos by *in situ* hybridization (M, N). **(A-L)** Triple labeling experiment to detect Runx1 (A, E, I; C, G, K: green), β-galactosidase/Pea3 (B, F, J; C, G, K: red) and Isl1 (D, H, L) expression. Sections are oriented in a rostral to caudal gradient, with (A-D) being the most rostral and (I-L) the most caudal sections at the level of C7/8. Note Runx1 expression in some motor neurons immediately rostrally of the Pea3 expressing motor neuron pool (A-C). Within the Pea3 positive motor neuron pool only ventrally located motor neurons express Runx1 (E-G), while motor neurons of the caudal part of the Pea3 motor neuron pool do not express Runx1 (I-K). **(M, N)** Expression of *Pea3* and the Runx partner subunit encoding gene *Cbfβ* in C7/8 motor neuron pools and DRG on serial sections.

Moreover, as would be predicted for Runx1 to be transcriptionally active, *Cbfβ* encoding the partner subunit of the Runx transcription factors is not only expressed within the DRG (where it provides the partner subunit for Runx1 and Runx3 in subpopulations of sensory neurons) but also in a defined subset of motor neurons, which is present at the same segmental level as the *Pea3* expressing motor neurons (Figure 33M, N).

In addition, the observed interaction of Runx3 and Er81 *in vitro* appears to be of functional significance as could be demonstrated by luciferase reporter assays measuring transcriptional transactivation potentials of ETS transcription factors of the Pea3 subfamily transfected either alone or cotransfected together with Runx3 (T. Portmann, diploma thesis 2004). Interestingly, while cotransfection of Runx3 had only minor effects on the transactivation capacity of Pea3, it had a strong synergistic effect on the transactivation potential of Er81, as the values of normalized relative luciferase activity increased by more than 5-fold in this assay. Importantly, when reporter plasmids lacked functional Runx DNA binding sites, coexpression of Runx3 had either no effect or even exerted a repressive effect on measured luciferase activities (T. Portmann, diploma thesis 2004). The latter could be explained by titration of Er81 through binding of Runx3 to Er81, which could occur due to absence of appropriate Runx DNA binding sites not on the reporter plasmid but elsewhere, therefore leading to a decrease in reporter activity. Similarly, it has been shown that the synergistic effect exerted by Runx2 and Ets1 on the activity of the *osteopontin/secreted phosphoprotein 1* (*Opn/Spp1*) promoter *in vitro* was abolished when either the ETS or Runx DNA recognition sequences were mutated, and that direct interaction of Runx2 with Ets1 required DNA binding of both transcription factors (Sato et al., 1998).

Together, these findings demonstrate that Runx3 and Er81 as well as Runx1 and Pea3 interact, at least *in vitro* on the basis of co-immunoprecipitation assays. Since Runx3 and Er81 are coexpressed within proprioceptive DRG sensory neurons, and also functionally interact in luciferase reporter assays *in vitro*, and Runx1 is coexpressed with Pea3 in a ventral subpopulation of motor neurons of the Pea3 positive motor neuron pool, it is attractive to speculate that Runx and ETS transcription factors of the Pea3 subfamily

specifically interact *in vivo* to control neuronal subpopulation specific gene expression during embryonic development. It would be interesting to determine whether the transcriptional transactivation potential of Pea3 could be specifically increased by coexpression of Runx1 in luciferase reporter assays *in vitro*, similar to the observed effect of Runx3 coexpression on the transactivation capacity of Er81.

## RUNX3 AND ER81 INTERACT GENETICALLY *IN VIVO*

To further explore the possibility of a functional interaction of Runx3 and Er81 *in vivo*, we characterized Runx3 expression in DRG of *Er81*$^{-/-}$ embryos. In contrast to Runx3, which is expressed soon after proprioceptive sensory neurons have been generated, expression of Er81 is induced in proprioceptive neurons by about E13 by target-derived NT3 (chapter 3 of this thesis). Thus, from E13 onwards, Er81 and Runx3 are coexpressed and could potentially interact to cooperate to control aspects of late proprioceptive DRG neuron specification (Figure 34).

Therefore, we focused our analysis of Runx3 expression in *Er81* mutants on two different developmental time points, namely E14.5, shortly after onset of Er81 expression, but before the first phenotypic abnormalities become detectable in *Er81*$^{-/-}$ embryos (Arber et al., 2000), and secondly a late time point, E17.5. While there was no difference in the number of Runx3 expressing neurons or the intensity of Runx3 expression in E14.5 *Er81*$^{-/-}$ DRG (data not shown), we detected a significant decrease of about 50% in the number of Runx3 positive proprioceptive sensory neurons in E17.5 *Er81*$^{-/-}$ DRG compared to littermate controls (Figure 35A, B, D, E). Importantly, the total number of proprioceptive DRG sensory neurons was not changed in *Er81*$^{-/-}$ mice (Figure 35C, F; Arber et al., 2000), indicating that the reduction in Runx3 positive DRG neurons was not due to a developmental loss of sensory neurons.

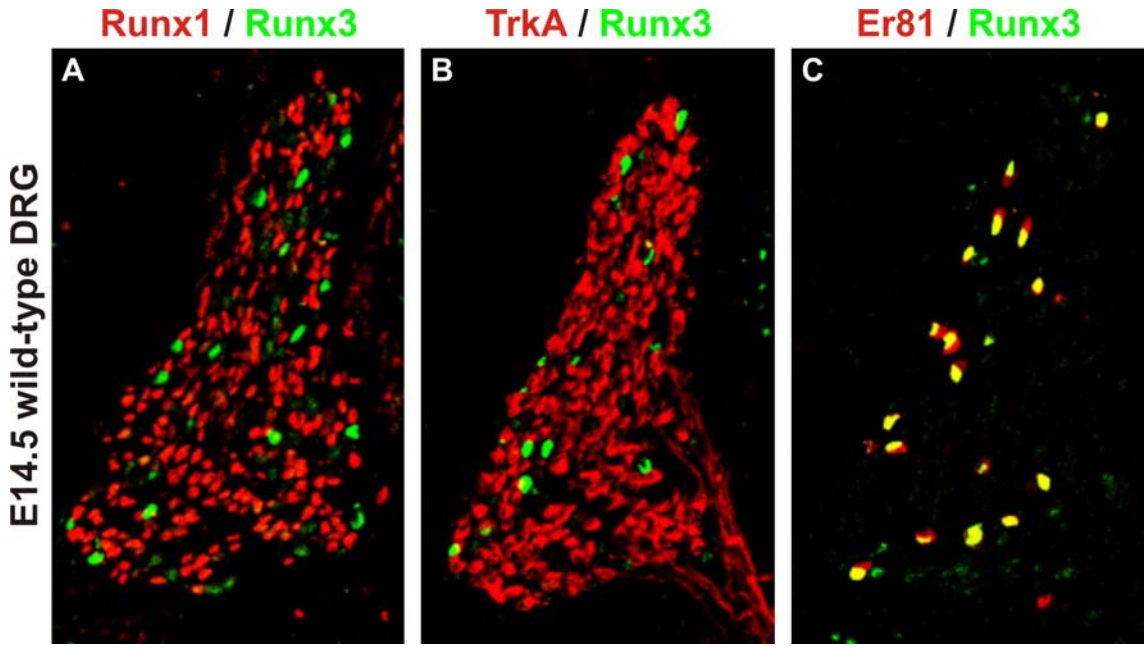


**Figure 34.** Runx3 is coexpressed with Er81 in proprioceptive DRG sensory neurons

Immunohistochemical analysis of Runx3 (green) expression on sections of E14.5 wild-type brachial DRG. (A) Runx1 and Runx3 are expressed by distinct, non-overlapping subpopulations of DRG sensory neurons. (B) Runx3 is not expressed in TrkA positive cutaneous DRG neurons. (C) Runx3 is coexpressed with Er81, a member of the Pea3 subfamily of ETS transcription factors, in proprioceptive DRG neurons.

To determine whether the loss of Runx3 expression in proprioceptive sensory neurons of *Er81*$^{-/-}$ embryos would progressively increase during development, we also analyzed the level of Runx3 expression in newborn *Er81*$^{-/-}$ mice. However, there was no further increase in the observed loss of Runx3 expression, as Runx3 continued to be expressed in about 50% of proprioceptive DRG sensory neurons in P0 *Er81*$^{-/-}$ mice (data not shown). Thus, Er81 activity is specifically required to maintain normal Runx3 expression in a defined subset of proprioceptive DRG sensory neurons, while it is dispensable for maintenance of Runx3 expression in the other 50% of proprioceptive sensory neurons.

The selective downregulation of Runx3 expression in about 50% of proprioceptive DRG neurons in absence of *Er81* activity is intriguing, as some of the defects observed in *Er81*$^{-/-}$ mice might reflect in part loss of Runx3 function or at least might depend partly on synergistic activities of Runx3 and Er81.

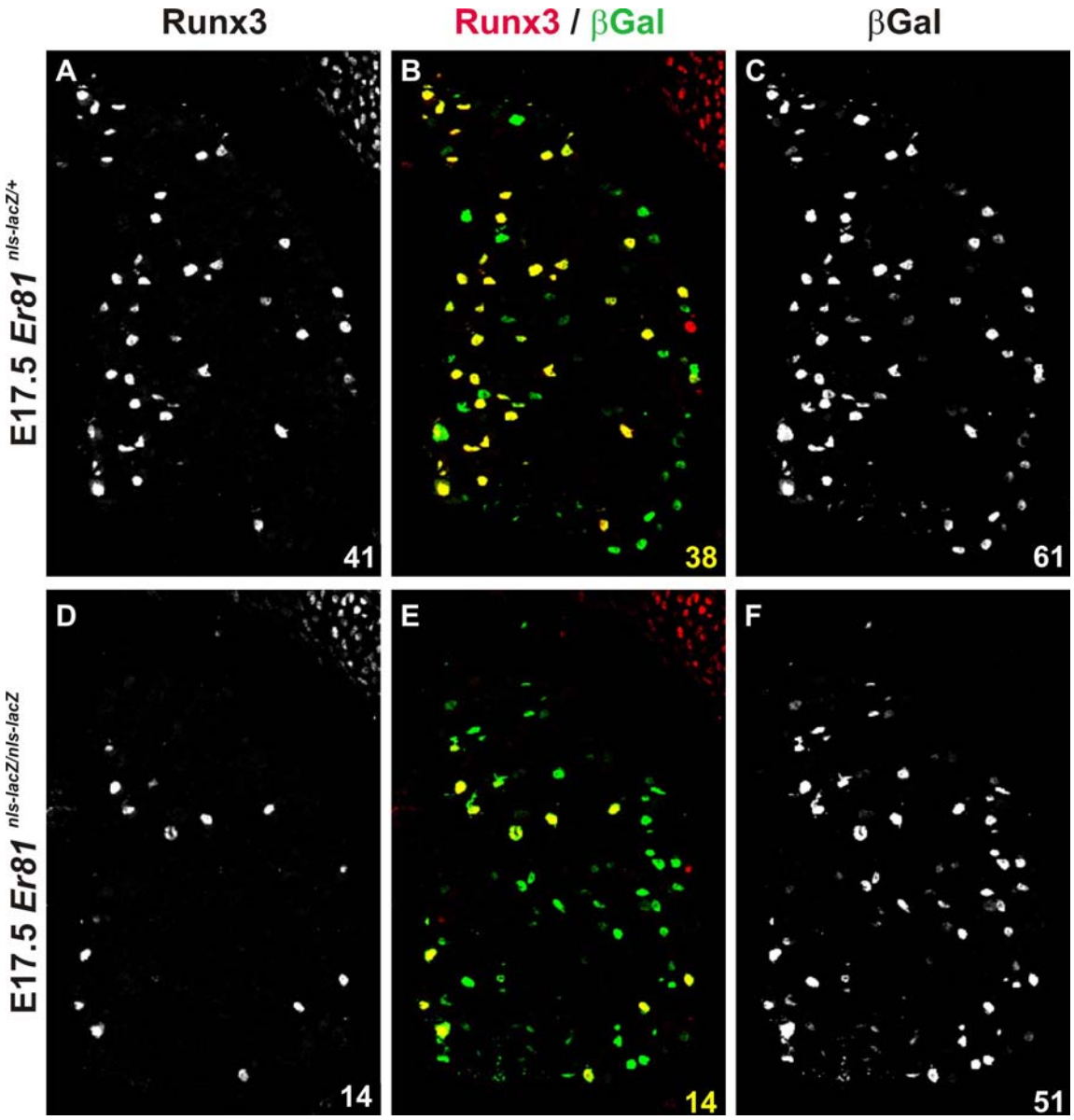


**Figure 35.** *Er81* is required to maintain Runx3 expression in a subset of DRG neurons

Immunohistochemical analysis of E17.5 lumbar L4 DRG in *Er81*$^{nls\text{-}lacZ/+}$ (A-C) and *Er81*$^{nls\text{-}lacZ/nls\text{-}lacZ}$ embryos. Double labeling experiment to detect Runx3 (A, D; B, E: red), and β-galactosidase expression (B, E: green; C, F). **(A-F)** Insets indicate number of Runx3 positive (A, D), β-galactosidase positive (C, F) or Runx3/β-galactosidase double positive (B, E: yellow) neurons. Note reduction in Runx3 expression in a subpopulation of proprioceptive DRG neurons in homozygous *Er81* mutant embryos (compare A, D and B, E) while the total number of β-galactosidase expressing neurons is not significantly reduced (compare B, E and C, F).

To address the potential contribution of Runx3 to the *Er81* knockout phenotype, we attempted to rescue *Er81*$^{-/-}$ mice by a *Runx3* gain of function approach. To this end, the

*Tau*$^{Runx3}$ allele driving expression of Runx3 and β-galactosidase from a bi-cistronic message (see chapter 7 of this thesis) was introduced into the *Er81*$^{ETS}$ homozygous mutant background (Arber et al., 2000). Ectopic Runx3 expression was activated by Cre recombinase expressed from the *Isl1* locus using *Isl1*$^{Cre}$ mice (Srinivas et al., 2001).
In *Er81*$^{-/-}$ mice Ia proprioceptive sensory neurons, which express the proprioceptive marker parvalbumin, fail to establish a ventral termination zone within the spinal cord and instead terminate prematurely in the region of the intermediate spinal cord (Arber et al., 2000). Before testing whether maintaining Runx3 expression in *Er81*$^{-/-}$ proprioceptive sensory neurons would affect central proprioceptive afferent projections in *Er81*$^{-/-}$ embryos, we first characterized the effect of Runx3 overexpression in a wild-type genetic background (Figure 36). In *Tau*$^{Runx3/+}$/*Isl1*$^{Cre}$ embryos analyzed at E17.5 no major phenotypic alterations of proprioceptive sensory neurons could be detected compared to wild-type littermate controls, neither with respect to Er81 expression (Figure 36A, D), nor with respect to central projections of proprioceptive DRG neurons visualized by parvalbumin immunocytochemistry (Figure 36C, F).

Next, we assessed the consequences of Runx3 overexpression in *Er81*$^{-/-}$ mice, focusing on the central projection defect of proprioceptive sensory neurons in *Er81*$^{-/-}$ embryos (Figure 37). While Runx3 expression was now maintained in all proprioceptive sensory neurons due to forced expression from the *tau* locus, this was not sufficient to promote growth of Ia proprioceptive afferent projections into the ventral horn of the spinal cord in absence of *Er81* function (compare Figure 37B, E and C, F). Moreover, a second well-characterized phenotype of *Er81*$^{ETS}$ knockout mice, namely the reduction of parvalbumin levels in proprioceptive DRG neurons (Arber et al., 2000), was also not rescued by Runx3 overexpression (data not shown). In contrast, as reported in chapter 7 of this thesis ectopic Runx3 expression in mechanoreceptive TrkB positive sensory neurons using the same genetic strategy led to a rapid downregulation in TrkB expression, thus demonstrating functional activity of ectopic Runx3 expressed from the *tau* locus.
Therefore, loss of Runx3 expression in about 50% of proprioceptive DRG neurons in *Er81*$^{-/-}$ mice does not appear to be a major causative factor with respect to the observed proprioceptive sensory neuron phenotype, as Runx3 was neither sufficient to restore

ventral projections of Ia proprioceptive afferents nor was it sufficient to maintain normal parvalbumin levels in proprioceptive DRG neurons.

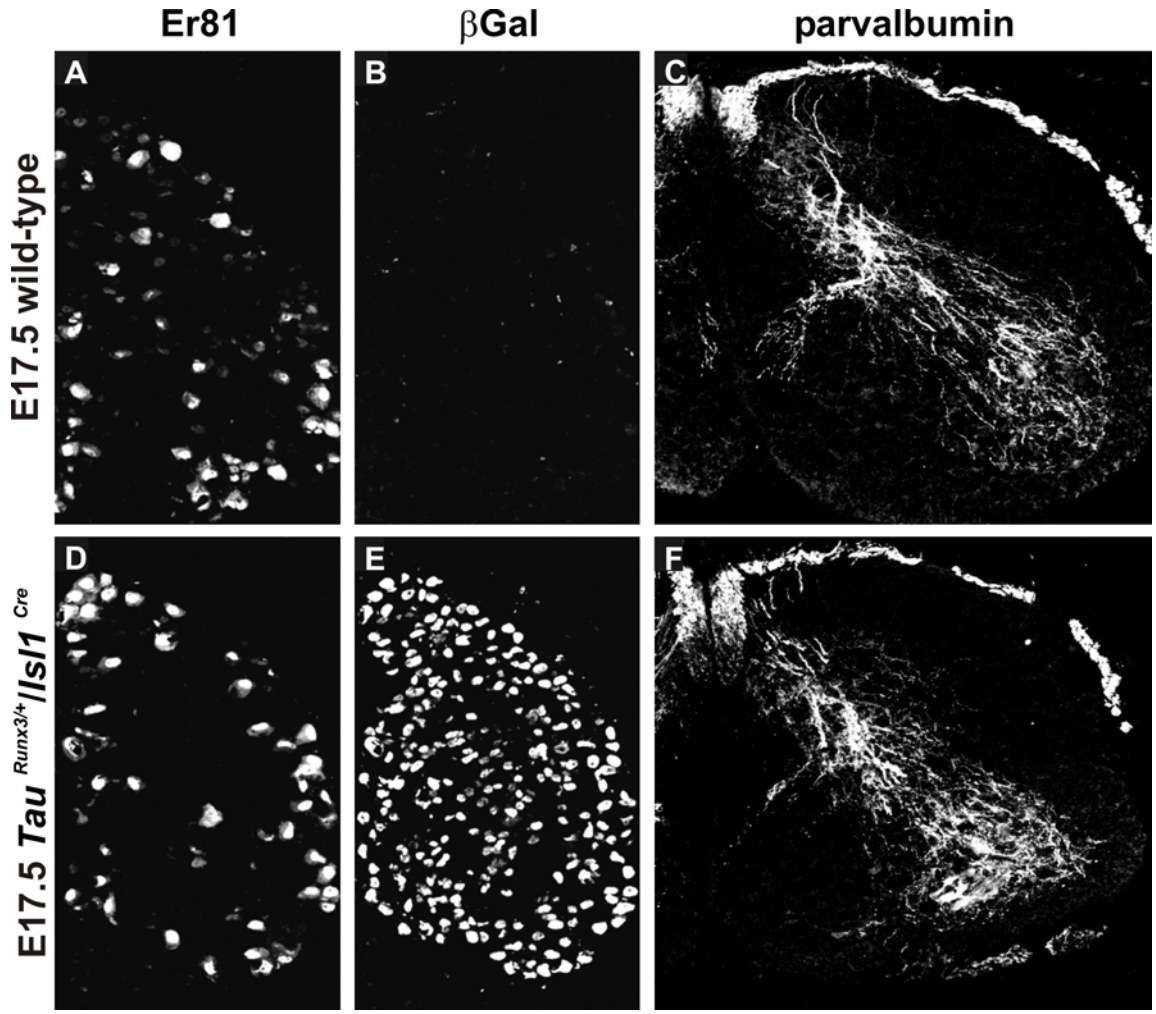


**Figure 36.** Proprioceptive DRG neuron character is not changed by Runx3 overexpression

Immunohistochemical analysis of E17.5 lumbar DRG (A, B, D, E) and spinal cord (C, F) in wild-type (A-C) and *Tau*$^{Runx3/+}$/*Isl1*$^{Cre}$ embryos (D-F) to detect Er81 (A, D), β-galactosidase derived from the *Tau*$^{Runx3}$ allele (B, E), or parvalbumin (C, F). Note that Runx3 overexpression neither alters Er81 expression, nor induces major changes in proprioceptive DRG sensory neuron projections.

However, this does not preclude a role for Runx3 in either of these processes, since Runx3 could still be required, while at the same time not being sufficient for establishment of a ventral termination zone of Ia proprioceptive afferents.

As we did not perform a detailed time course analysis concerning the downregulation of Runx3 in *Er81*$^{-/-}$ mice, these findings may not seem too surprising, since loss of Runx3

expression in a subpopulation of proprioceptive sensory neurons might reflect a late consequence of other phenotypic defects rather than being a primary cause of *Er81* deficiency.

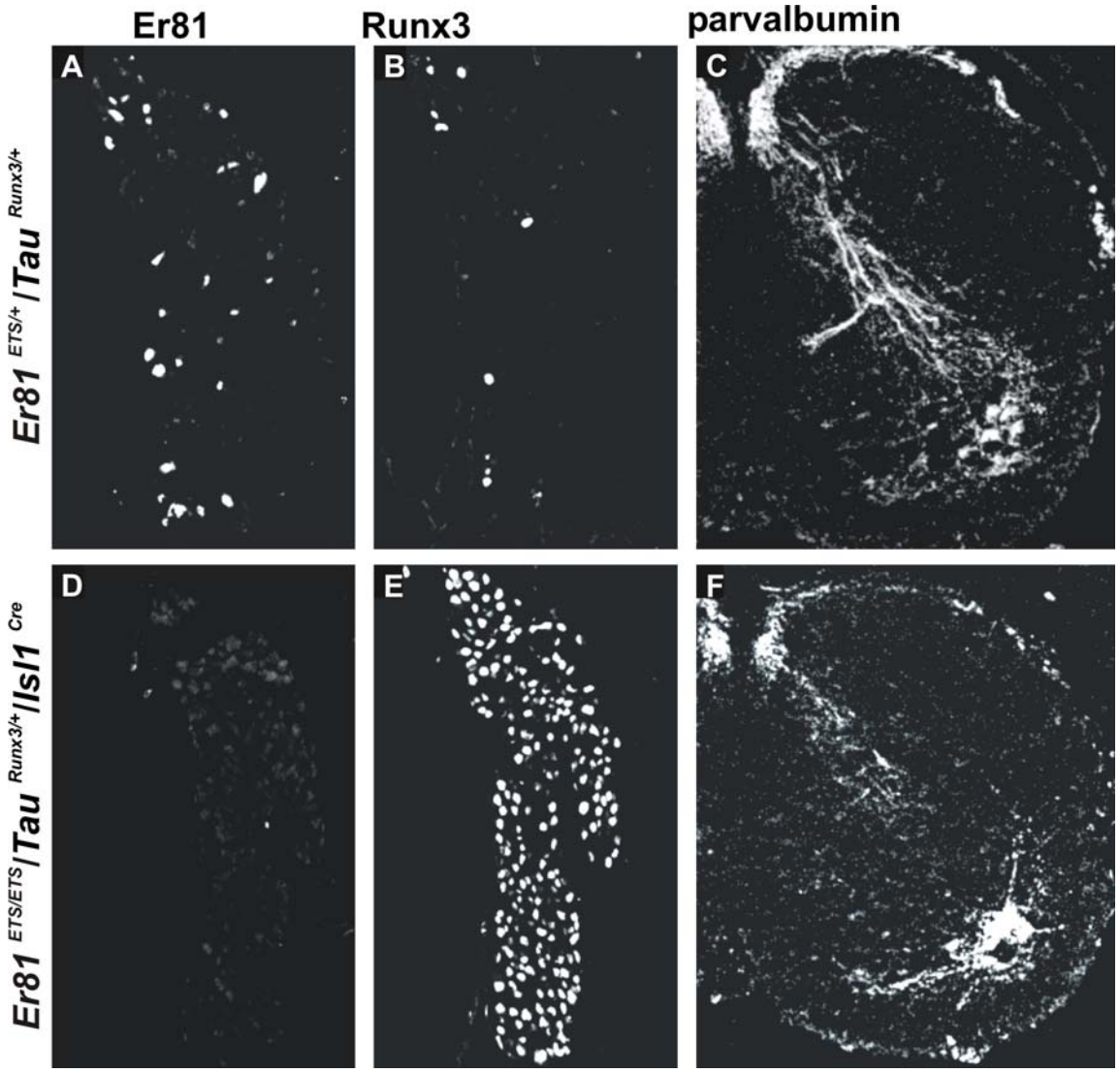


**Figure 37.** Runx3 overexpression does not rescue the *Er81*$^{-/-}$ sensory neuron phenotype

Immunohistochemical analysis of E17.5 lumbar L1 DRG (A, B, D, E) and spinal cord (C, F) in *Er81*$^{ETS/+}$/*Tau*$^{Runx3/+}$ (A-C) and *Er81*$^{ETS/ETS}$/*Tau*$^{Runx3/+}$/*Isl1*$^{Cre}$ embryos (D-F) to detect Er81 (A, D), Runx3 (B, E), or parvalbumin (C, F). Note that Runx3 overexpression is not sufficient to rescue the *Er81*$^{-/-}$ phenotype, neither with respect to parvalbumin levels in proprioceptive DRG sensory neurons (data not shown), nor with respect to the central projection defect of Ia proprioceptive DRG neurons, which terminate prematurely in the intermediate region of the spinal cord (compare C and F).

Moreover, the fact that DRG sensory neurons appear to predominantly express the proximal P2 promoter isoforms of *Runx* genes, which have not been reported to contain ETS DNA binding sites, in contrast to the distal P1 promoters, does not suggest a direct role for Er81 in controlling *Runx3* expression in proprioceptive sensory neurons.

# METHODS

## TRANSIENT TRANSFECTIONS OF COS-7 CELLS

Plasmids used for transient transfection of COS-7 cells (American Type Culture Collection) were derivatives of the pcDNA3 mammalian expression vector (Invitrogen) containing inserts of either of the following cDNAs encoding: proximal P2 promoter isoform of Runx1 (W29200) or Runx3 (BC013362), N-terminally Myc-tagged Er81 (gift from J. A. Hassell), or N-terminally Myc-tagged Ets1 (BI105114). COS-7 cells grown in 10 cm dishes to about 70-95% confluency in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum were transiently transfected or cotransfected with 5-6 μg plasmid DNA in total per dish using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One 10 cm dish of transfected COS-7 cells was used as input for two co-immunoprecipitation experiments.

## CO-IMMUNOPRECIPITATION ASSAYS

Transiently (co-)transfected COS-7 cells were harvested 36 hours after transfection and lysed in ice-cold TNN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40) in presence of protease and phosphatase inhibitors. Cell lysates were precleared with protein G-Sepharose beads (Pharmacia) for 30 minutes at 4°C and then immunoprecipitated with monoclonal mouse anti-Myc (Myc1-9E10.2) or mouse anti-Flag M2 (Stratagene) antibodies. Precipitated proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting using either of the following primary antibodies: rabbit anti-Runx1 (generated against a mouse

Runx1 peptide: GRASGMTSLSAELSSRL), rabbit anti-Runx3 (generated against a 6xHis tagged carboxy-terminal fusion protein of Runx3; amino acids 187-415) or rabbit anti-Runx3 (generated against a mouse Runx3 peptide: MSAAFPYSATPS). Following incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Pharmacia) bands were visualized using Lumi-Light Plus Western blotting substrate (Roche).

## ANIMALS

*Er81*$^{ETS}$, *Er81*$^{nls\text{-}lacZ}$ (Arber et al., 2000), *Pea3*$^{nls\text{-}lacZ}$ (Livet et al., 2002) and *Isl1*$^{Cre}$ (Srinivas et al., 2001) mouse strains have been described previously. Generation of *Tau*$^{Runx3}$ mice is described in chapter 7 of this thesis.

## *IN SITU* HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

For *in situ* hybridization analysis, cryostat sections were hybridized with digoxigenin labeled antisense riboprobes against mouse *CBFβ* (BC006763) or mouse *Pea3* as previously described (Schaeren-Wiemers and Gerfin-Moser; 1993). Antibodies used comprise: goat anti-β-galactosidase (Arnel), guinea pig anti-Isl1 (Arber et al., 2000), rabbit anti-Runx1 (generated against a mouse Runx1 peptide: GRASGMTSLSAELSSRL), guinea pig anti-Runx3 (generated against a mouse Runx3 peptide: MSAAFPYSATPS), rabbit anti-Runx3 (generated against a 6xHis tagged carboxy-terminal fusion protein of Runx3; amino acids 187-415), and rabbit anti-Runx3 (generated against a mouse Runx3 peptide: MSAAFPYSATPS). Cryostat sections were processed for immunohistochemistry as previously described (Arber et al, 2000) using fluorophore-conjugated secondary antibodies (Molecular Probes). Images were collected with an Olympus confocal microscope or for *in situ* hybridization experiments with a RT-SPOT camera (Diagnostics Instruments).

# CHAPTER 9

# GENERAL DISCUSSION AND PERSPECTIVES

## MOLECULAR MECHANISMS OF PROPRIOCEPTIVE NEURON SPECIFICATION

The aim of this doctoral thesis was to contribute to the identification and characterization of the molecular mechanisms leading to proprioceptive DRG sensory neuron specification during embryonic development. The two main findings of this work have been summarized in chapters 3 and 7 of this thesis and will be discussed in the following sections with respect to the current understanding of sensory neuron differentiation.

## IMPACT OF TARGET-DERIVED FACTORS ON NEURONAL CIRCUIT ASSEMBLY

Chapter 3 focused on the identification of the peripheral signal responsible for the induction of expression of the ETS transcription factor Er81 in proprioceptive DRG sensory neurons. Er81 has been shown to play a key role in late sensory-motor circuit establishment, as in mice lacking *Er81* function Ia proprioceptive sensory neurons fail to project to their central targets in the ventral horn of the spinal cord, where they normally branch and form direct monosynaptic connections with α-motor neurons projecting to the same or functionally related skeletal limb muscles in the periphery (Arber et al., 2000). Based on earlier studies in the chick embryo, it had already been shown that the induction of two ETS transcription factors of the Pea3 subfamily, namely Er81 and Pea3, in proprioceptive DRG sensory neurons and spinal motor neurons relies on target-derived molecules acting on the axonal growth cones of sensory and motor axons as these reach the base of the limb and are about to invade their future skeletal target muscles (Lin et al., 1998). However, the identity of the responsible peripheral signals remained elusive. Linking the induction of important transcriptional regulators, in this case of ETS transcription factors of the Pea3 subfamily, to preceding steps in neuronal circuit establishment provides an elegant means by with the initiation of subsequent steps in differentiation become critically dependent to functional accomplishment of earlier steps in neuronal differentiation. It is intriguing that the peripheral signal for induction of Er81 expression has been found to be a neurotrophin, NT3, which at the same time is required for mediating survival of TrkC positive proprioceptive sensory neurons. Thus, induction of further steps in sensory neuron differentiation like axonal invasion of the ventral horn, branching and establishment of functional synapses of correct specificity with

postsynaptic target motor neurons critically rely on the interplay of sensory neurons with their peripheral target muscles. The advantage of such a binary system of crosstalk between neurons and their prospective targets is clearly that only those neurons having correctly navigated to the distant targets in the periphery will gain the possibility to survive and "receive instructions" to induce subsequent steps in differentiation. Moreover, taking this concept further, also the target cells will in turn receive instructive signals from their neuronal partners allowing them to undergo further differentiation. Accordingly, muscle spindle induction has been shown to rely on molecular signals provided by proprioceptive sensory neurons expressing at their axon terminals a specific isoform of neuregulin 1 (Nrg1) characterized by an extracellular immunoglobulin (Ig)-like domain (Ig-Nrg1; Hippenmeyer et al., 2002). In mice lacking all Nrg1 isoforms muscle spindle induction no longer takes place, whereas mice still expressing the Ig-Nrg1 isoform develop normal muscle spindles, thus demonstrating that Ig-Nrg1 expressed by proprioceptive sensory neurons is sufficient for muscle spindle induction.

While the peripheral signal responsible for induction of Pea3 in spinal α-motor neurons had been already identified as being the muscle target-derived neurotrophic factor GDNF (Haase et al., 2002), acting on GFRα1/Ret positive motor terminals, the identity of the peripheral signal leading to induction of ETS transcription factor expression in proprioceptive DRG sensory neurons remained unknown. Therefore, the identification of NT3 acting as a peripheral target-derived factor to induce Er81 expression in proprioceptive sensory neurons, as described in chapter 3 of this thesis (Patel et al., 2003), adds an important aspect to the current understanding of regulation of ETS transcription factor expression in differentiating neurons. Moreover, it highlights a perhaps more general function of neurotrophic factors in controlling late steps of neuronal differentiation in parallel to their well known role in mediating neuronal survival during development. It is likely to assume that target-derived neurotrophic factors contribute to the induction of late steps in neuronal differentiation not solely by inducing ETS transcription factor expression but also by initiating additional yet unknown molecular signaling cascades. Along this line, it is interesting that in contrast to *Er81*$^{-/-}$ mice, in

which muscle spindle differentiation initially proceeds normally, muscle spindle induction in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice appears to be severely impaired (see chapter 3).

One possible way to further clarify the impact of NT3 signaling on proprioceptive sensory neuron differentiation would be to rescue the lack of Er81 expression in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice by overexpressing *Er81* in DRG sensory neurons. Theoretically, such an experiment could be easily achieved with a gain-of-function approach similar to the one described in chapter 7 of this thesis for misexpression of the transcription factor Runx3. Moreover, mice conditionally misexpressing *Er81* in all DRG sensory neurons would provide a potential tool for identifying Er81 target genes in differentiating sensory neurons, which so far remain elusive, by comparing gene expression values from *Er81* overexpressing DRG with wild-type and *Er81*$^{-/-}$ DRG. As Er81 is normally only expressed by proprioceptive DRG sensory neurons, which constitute a minority of only 10-20% of all DRG neurons, identification of Er81 target genes would otherwise require to selectively isolate proprioceptive DRG neurons from wild-type and *Er81*$^{-/-}$ embryos prior to gene expression profiling instead of using whole ganglia, in which about 80% of the neuronally expressed genes are likely to not be affected by loss of *Er81* function.

In conclusion, in future experiments, it will be important to determine which factors are responsible for mediating NT3 signaling and mechanisms of action in proprioceptive DRG sensory neurons. In particular, it will be necessary to identify the cell-intrinsic factors present within proprioceptive DRG sensory neurons, but not for example within spinal α-motor neurons, which also express TrkC receptor and do not respond to NT3 signaling with induction of Er81 expression. It is clear, that intrinsic differences must exist beyond the expression of certain receptor subtypes to allow initiation of specific signaling cascades in response to identical target-derived factors encountered by axons of different neuronal subpopulations along their way to their final target destinations.

## THE ROLE OF RUNX3 IN PROPRIOCEPTIVE DRG NEURON SPECIFICATION

In an Affymetrix microarray based screen described in chapter 5 of this thesis we identified the runt related transcription factors Runx1 and Runx3 as being specifically

expressed in different subpopulation of differentiating DRG neurons during embryonic development. While Runx1 is expressed within a subpopulation of TrkA positive cutaneous DRG neurons, Runx3 is expressed selectively by TrkC positive proprioceptive sensory neurons.

As discussed in chapter 7 of this thesis, Runx3 is required during early sensory neuron differentiation to determine the neurotrophic factor receptor phenotype expressed by developing sensory neurons, thereby contributing to the consolidation of proprioceptive sensory neuron identity through concomitant repression of alternative mechanoreceptive sensory neuron phenotypes. While it had previously been shown that neurotrophic factor receptor expression is not only required for mediating neuronal survival but also is associated with neuronal fate acquisition and specification of neuronal subpopulation specific phenotypic traits such as axonal projections and target selection (Patel et al., 2000; Hellard et al., 2004; Moqrich et al., 2004), the molecular mechanisms controlling neurotrophic factor receptor expression in different neuronal subpopulations are not well understood. Interestingly, early in development soon after their generation many DRG sensory neurons transiently express two neurotrophic factor receptors. This feature might be indicative of a short phase of early developmental plasticity with respect to the acquisition of a given neuronal fate. Depending on cell-intrinsic and perhaps also cell extrinsic factors, such as limiting amounts of neurotrophic factors, the early neurotrophic factor receptor coexpression rapidly segregates into distinct sensory neuron subpopulations expressing only a single neurotrophic factor receptor. As shown in chapter 7 of this thesis, Runx3 expression is almost exclusively associated with a TrkC single positive state in proprioceptive DRG sensory neurons and Runx3 misexpression leads to a complete absence of TrkB expression in DRG sensory neurons. Conversely, in *Runx3*$^{-/-}$ DRG, TrkC single positive proprioceptive neurons cannot be detected, since all presumptive proprioceptive sensory neurons now express a hybrid TrkB/TrkC phenotype. This is also reflected by an over 100% increase in the total number of TrkB expressing DRG sensory neurons. Moreover, Runx3 acts cell autonomously to repress TrkB expression in DRG sensory neurons and appears to promote proprioceptive sensory neuron specification cell-intrinsically through negative regulation of acquisition of an

alternative mechanoreceptive neuronal fate. Together, these results therefore provide genetic evidence for a role of Runx3 in determining the neurotrophic factor receptor phenotype expressed by developing DRG sensory neurons.

How can these findings be reconciled with the previously reported phenotypes of *Runx3* knockout mice? The first study addressing the role of Runx3 in DRG sensory neurons specification (Levanon et al., 2002) revealed that Runx3 is required for survival of TrkC expressing proprioceptive sensory neurons. While initial onset of TrkC expression occurred normally in *Runx3*$^{-/-}$ DRG, in accordance with TrkC starting to be expressed slightly before Runx3 at E10.5 (our unpublished observation; Farinas et al., 1998), TrkC expression was rapidly lost in absence of Runx3 activity and was no longer detectable from E14 onwards. Moreover, expression of the other neurotrophin receptors, TrkA and TrkB, as well as expression of Runx1 were not altered in cervical DRG analyzed in E15.5 *Runx3*$^{-/-}$ embryos. These findings prompted the authors to propose a role for Runx3 in positively regulating TrkC expression and/or being required for suppression of a negative regulator and are thus in good agreement with our findings. According to our observations, in absence of Runx3 function proprioceptive sensory neurons are eliminated during embryonic development as they fail to progress from a TrkB/TrkC hybrid phenotype to a TrkC single positive state. Since early neurotrophic factor receptor expression in DRG neurons was not analyzed at the time of normal onset of Runx3 expression in neither of the two previous *Runx3* knockout studies, the early function of Runx3 to selectively repress TrkB expression within TrkC proprioceptive neurons might have been missed.

The second report on Runx3 function in DRG sensory neurons did however not reveal a role for Runx3 in control of neurotrophic factor receptor expression (Inoue et al., 2002). Neither a reduction in the number of proprioceptive DRG neurons nor in TrkC expression was observed in newborn *Runx3*$^{-/-}$ mice. In contrast, the authors observed a selective defect in neurite outgrowth of proprioceptive DRG neurons. While initial rostro-caudal extension of proprioceptive axons in the dorsal funiculus appeared to occur normally in *Runx3*$^{-/-}$ embryos subsequent invasion of the spinal cord by collateral sprouting was severely impaired and axonal projections towards the intermediate region of the spinal

cord were basically absent. As a molecular explanation for the observed phenotype the authors proposed two models for Runx3 function in proprioceptive DRG sensory neurons. In the first model it is suggested that Runx3 controls the ability of proprioceptive axonal growth cones to correctly sample their local environment, allowing correct axonal navigation through the dorsal spinal cord to reach the intermediate region of the spinal cord. In the second model, Runx3 would be required for promoting general axonal outgrowth, as it was observed that DRG explants isolated from *Runx3*$^{-/-}$ embryos and cultured in presence of NT3 displayed significantly reduced neurite outgrowth *in vitro* compared to wild-type control explants.

However, a third potential explanation for the proprioceptive axon guidance defect observed in *Runx3*$^{-/-}$ embryos, which is not discussed by the authors, might be a selective defect in proprioceptive afferent innervation of peripheral targets. Interestingly, it is believed that the occurrence of so-called waiting periods before final target invasion might reflect a dependency on extrinsic factors, which act as instructive signals required to initiate further steps in neuronal circuit assembly. Similarly, the onset of axonal collateral sprouting to innervate the spinal cord occurs about E12 when proprioceptive axon terminals have reached their peripheral targets in the limb and begin to invade skeletal target muscles. Moreover, this coincides with the developmental onset of expression of the ETS transcription factor Er81 in proprioceptive sensory neurons. Interestingly, in *Runx3* mutants, in which proprioceptive neurons survive, expression of Er81, which is induced by target-derived NT3 (see chapter 3 of this thesis), still occurs. These findings suggest that proprioceptive neurons are capable of projecting to their normal peripheral skeletal target muscles in absence of Runx3 activity. However, it is possible that Runx3 might act as a permissive factor to ensure and coordinate correct integration of peripheral target-derived signals controlling subsequent invasion of central targets within the spinal cord. It would be interesting to know whether progression from a hybrid TrkB/TrkC phenotype to a TrkC single positive state, which is defective in our *Runx3* mutants as described in chapter 7, represents a prerequisite for correct interpretation of target-derived signals, especially since NT3 signals not only through its preferred receptor TrkC but also is a ligand for TrkB (Strohmaier et al., 1996; Farinas, 1998).

To address these and other potential late functions of Runx3 in more detail it would be necessary to carefully study the effects of conditional elimination of *Runx3* at later developmental time points, which could be accomplished by analysis of conditional *Runx3* mutants carrying a floxed *Runx3* allele crossed with suitable Cre driver mouse lines such as $PV^{Cre}$ mice, which express Cre recombinase under the control of the *parvalbumin* (*PV*) locus (Hippenmeyer et al., 2005). In addition, using the $Tau^{Runx3}$ mice described in chapter 7 of this thesis crossed with late expressing Cre driver lines such as $PV^{Cre}$ mice would allow to selectively overexpress Runx3 in proprioceptive or other subpopulations of sensory neurons after the time of consolidation of neurotrophic factor receptor expression controlled by Runx3. While $PV^{Cre}$ induced recombination would be too late for an analysis of a role of Runx3 in controlling proprioceptive axon collateral invasion into the spinal cord, it clearly would be very useful to address the question whether and how Runx3 and Er81 might interact functionally to control late steps of sensory-motor circuit establishment and to identify some of their common downstream target genes. This would be particularly interesting as there is evidence suggesting synergistic functions of Runx and Ets transcription factors during cellular differentiation and lineage determination in other biological systems (Li et al., 2000; Perry et al., 2002). Moreover, Runx3 and Er81 functionally interact *in vitro* and Runx3 expression is specifically lost in about 50% of proprioceptive DRG neurons in $Er81^{-/-}$ mice (see chapter 8 of this thesis). Thus, gain-of-function experiments together with the late conditional elimination of *Runx3* would be very informative for dissecting the different functions of Runx3 in proprioceptive DRG sensory neuron specification.

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# APPENDIX

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