Homeobox gene expression in adult dorsal root ganglia: Is regeneration a recapitulation of development?

Expressie van homeoboxgenen in volwassen dorsale streng ganglia: Is regeneratie een recapitulatie van ontwikkeling?

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op

dinsdag 29 april 2003 des middags te 16.15 uur

door

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geboren op 13 november 1975 te Utrecht

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Financial support for the publication of this thesis by van Leersum Fonds KNAW

Cover: C.F. Vogelaar

Print: Optima Grafische Communicatie Rotterdam

ISBN: 90-6734-197-5

“Rien de grand ne se fait en ce monde sans passion, sans entêtement. Qui ne se dépense pas ne s’enrichit jamais.”

Henri Troyat
Les Semailles et les Moissons:
La Rencontre, 1958

Voor Loes
Voor oma

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PREFACE

The nervous system is a highly complex integration of individual classes of neurons, interconnected through their fibers. The cell bodies of these neurons are located in defined structures within the adult nervous system. These structures are generated during embryonic development. Progenitor cells from the neural tube migrate to defined places and differentiate to become specific neurons in defined neuronal structures. During the period of neurite outgrowth their fibers follow specific routes to connect with target cells, for example other neurons, hormone secreting cells or peripheral tissues. Finally, the neurons obtain an adult neurotransmitter phenotype and their fibers obtain adult electrophysiological properties due to connections with glial cells that have developed simultaneously. By directing gene expression, homeobox genes are involved in patterning of the nervous system, in specification and differentiation of neurons, in directing outgrowth, pathfinding and maturation. The peripheral nervous system (PNS) is comprised of sensory ganglia and nerves that provide the central nervous system (CNS), i.e. the brain and spinal cord, with sensory information from the skin, muscles, joints and viscera. Unlike the central nervous system, the peripheral nervous system is able to regenerate after nerve injury. Regeneration seems to involve processes and gene expression programs similar to those used during development. This thesis will deal with the question whether regeneration is a recapitulation of development by investigating homeobox gene expression in the sciatic nerve crush model.

1. HOMEOBOX GENES AND NERVOUS SYSTEM DEVELOPMENT

1.1. The homeodomain binds to specific sequences in the DNA

Homeobox genes encode homeodomain proteins that act as transcription factors regulating the expression of specific target genes. The homeodomain is a DNA-binding domain that binds to the promoters of genes in a sequence-specific manner. This domain is generally 60 amino acids in size and is composed of 3 alpha helices organized in a helix-loop-helix configuration [Bürglin, 1994; Gehring et al, 1994]. The third helix is called the recognition helix and binds in the major groove of the DNA via amino that interact specifically with bases in the DNA (Figure 1 A&B). The N-terminal arm of homeodomain proteins reaches into the minor groove and a part of helix 2 also contributes to DNA binding. Most of the homeodomain proteins bind the core motif ATTA. The actual binding site of a specific homeodomain protein to the DNA depends on additional nucleotides bound by specific amino acids. For example, a very critical amino acid that determines sequence specificity is located at position 50 of the homeobox, in helix 3 [Bürglin, 1994; Gehring et al, 1994). To increase binding specificity, some homeodomain protein classes have additional DNA binding domains or interact with other (homeodomain) proteins via protein interaction domains (see section 1.2.2.).

1.2. Homeobox genes can be divided into classes

1.2.1. The Hox complex superclass

Homeobox genes were first identified in *Drosophila*, where so called homeotic mutations lead to transformations of body segments. The first homeotic gene identified was *Antennapedia* (*Antp*) the mutation of which resulted in the transformation of the antennae on the head of the fly into legs. More genes were found that bore great similarity with *Antp* in the region of the gene that was called the homeobox. Homeotic homeobox genes in *Drosophila* were present in two genetic clusters, called the *HOM-C* complex. Soon their vertebrate counterparts were identified, which were called *Hox* genes in the *Hox* complex [Gehring, 1994; Kenyon, 1994]. Figure 2A shows the alignment of the *Hox* complex with the *HOM-C* genes.

1.2.2. The dispersed superclass

Several other classes of homeobox genes were defined that are not present in the *Hox* complex but are largely dispersed in the genome. The subdivision into classes is based upon the presence of additional domains, the positions of introns and on certain amino acid characteristics [Banerjee-Basu and Baxevanis, 2001; Bürglin, 1994; Gehring et al, 1994]. These classes are outlined in figure 2B; the classes that are important for this thesis are described here. The **paired class** of homeobox genes contains a second DNA binding domain, the paired domain. In addition, some paired class proteins have an octapeptide motif between the paired domain and the homeodomain. All paired homeodomains have a serine residue at position 50. Some paired genes do not have a homeobox but only contain a paired box. The **paired-like class** of homeodomains is most similar to paired homeodomains, but lacks the serine 50 residue and is not associated with a paired domain. The **POU class** of homeodomain proteins contains two DNA binding domains: the POU-specific domain and the POU-homeodomain. Position 50 characteristically is a cysteine residue [He et al, 1989]. **LIM class** proteins contain in addition to the homeodomain two LIM motifs of about 60 amino acids, with conserved cysteine and histidine residues. These LIM motifs play major roles in protein-protein interactions [Hobert and Westphal, 2000]. Other classes include the **Msh class**, which does not have any conserved domains outside of the homeodomain; the **Gsh class** that maps outside the *Hox* complex but belongs to the same class and the **Zinc finger class**, consisting of 9 to 17 zinc fingers and 1 to 4 homeodomains [Bürglin, 1994; Gehring et al, 1994].

1.3. Homeobox sequences are conserved and are present in all species

The homeodomain is highly conserved: there is extensive sequence homology between homeodomain sequences (Figure 1C&D). Homeodomain proteins of the same class often share more than 85% of their homeodomain amino acids. There can be up to 57% homology between homeodomains of different classes [Banerjee-Basu and Baxevanis, 2001; Bürglin, 1994; Galliot et al, 1998; Gehring et al, 1994]. Outside the homeodomain the amino acid sequence is more variable, but the additional domains in certain classes are also conserved between members of the same class. This sequence homology, however, does not necessarily imply that members of the same class bind to the same target genes or have similar functions. One amino acid difference in the homeodomain can completely change the function of the protein. This was shown by Smith et al (1997), who demonstrated that the mutation of one single amino acid in the homeodomain of Brn3b was sufficient to convert Brn3b function into that of Brn3a. This also illustrates that a homeodomain protein can be identified based upon its homeodomain sequence. Moreover, at the DNA level, the homeobox sequences contain polymorphisms that do not encode amino acid differences, but can be used for identifying specific homeobox genes.

Homeobox genes are evolutionary conserved between species. They are present in all species ranging from yeast to hydra, from *C. elegans* (worm) to *Drosophila* to mammals, and have similar functions in embryonic development. More complex organisms, like mammals, need more complex regulation and therefore have a greater number of different homeobox genes. This can be due to duplications in the genome, which is the case for the *Hox* complex [Kenyon, 1994] (Figure 2A). After gene duplication the homologues are thought to diverge in function due to mutations leading to new close homologues. For example, there is only one *Brn3* homologue (*Unc-86*) in *C. elegans*, whereas there are 3 highly related *Brn3* homologues in the mouse - *Brn3a*, *Brn3b*, and *Brn3c* [Xiang et al, 1995]. Only one *Isl* gene is present in *Drosophila*, whereas vertebrates have *Isl1* and *–2* [Hobert and Westphal, 2000].

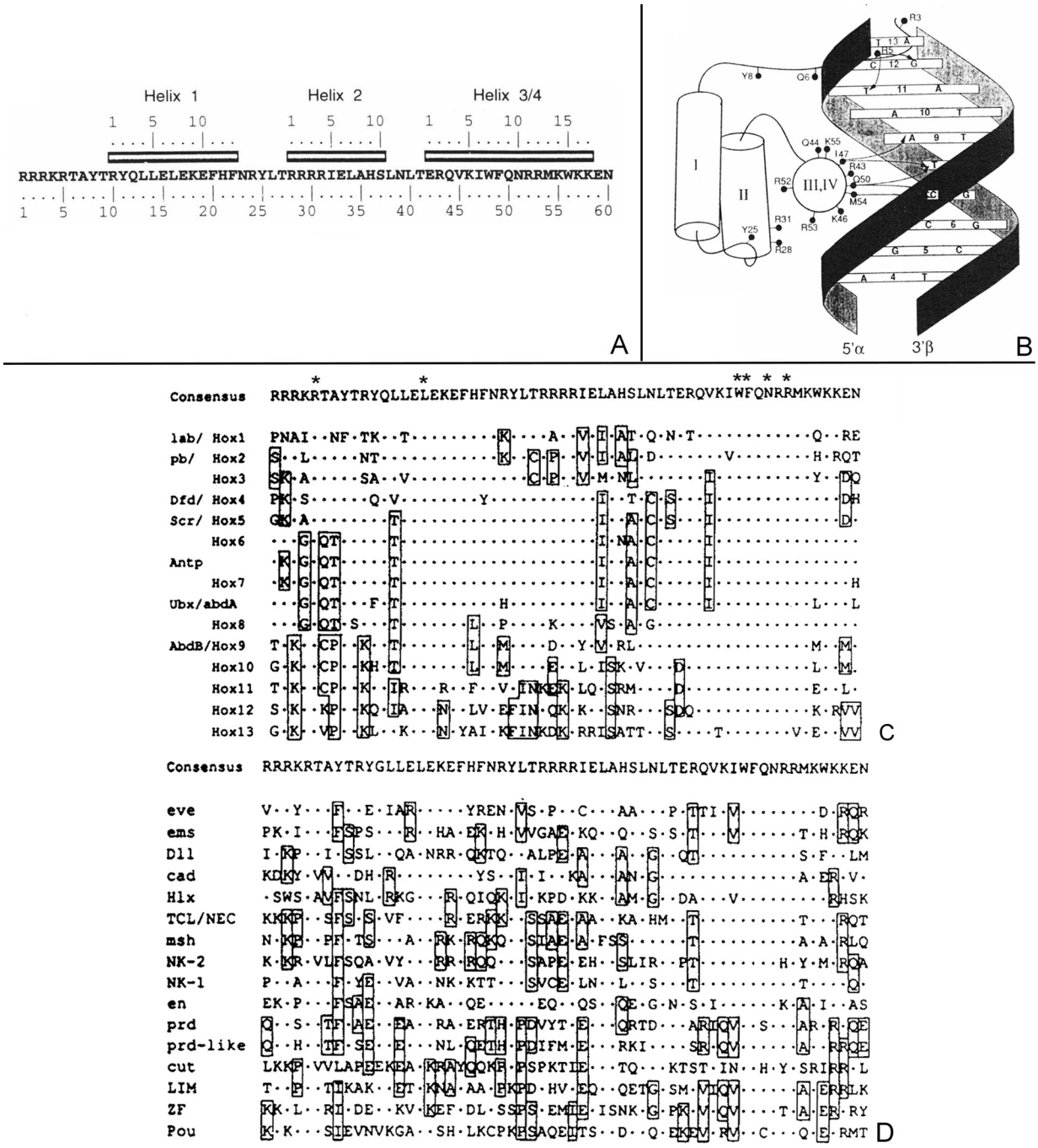


Figure 1: Structure of the homeodomain

(A) Amino acid consensus sequence based upon 346 homeodomain sequences. Standard numbering scheme and position of the three helices [Bürglin, 1994]. (B) Schematic drawing of the *Antennapedia* homeodomain-DNA complex. View along the axis of the recognition helix (III,IV). Amino acids that interact with the DNA are indicated by black circles. Nucleotide-specific contacts are indicated by arrows [Gehring, 1998]. (C) Alignment of the homeodomain families of the complex superclass and (D) the dispersed superclass with the consensus homeodomain sequence. Dots indicate positions where the proteins are similar to the consensus sequence. Boxed amino acids are shared between adjacent families [Gehring et al, 1994]. Amino acid residues that are shared among all families are marked with asterisks.

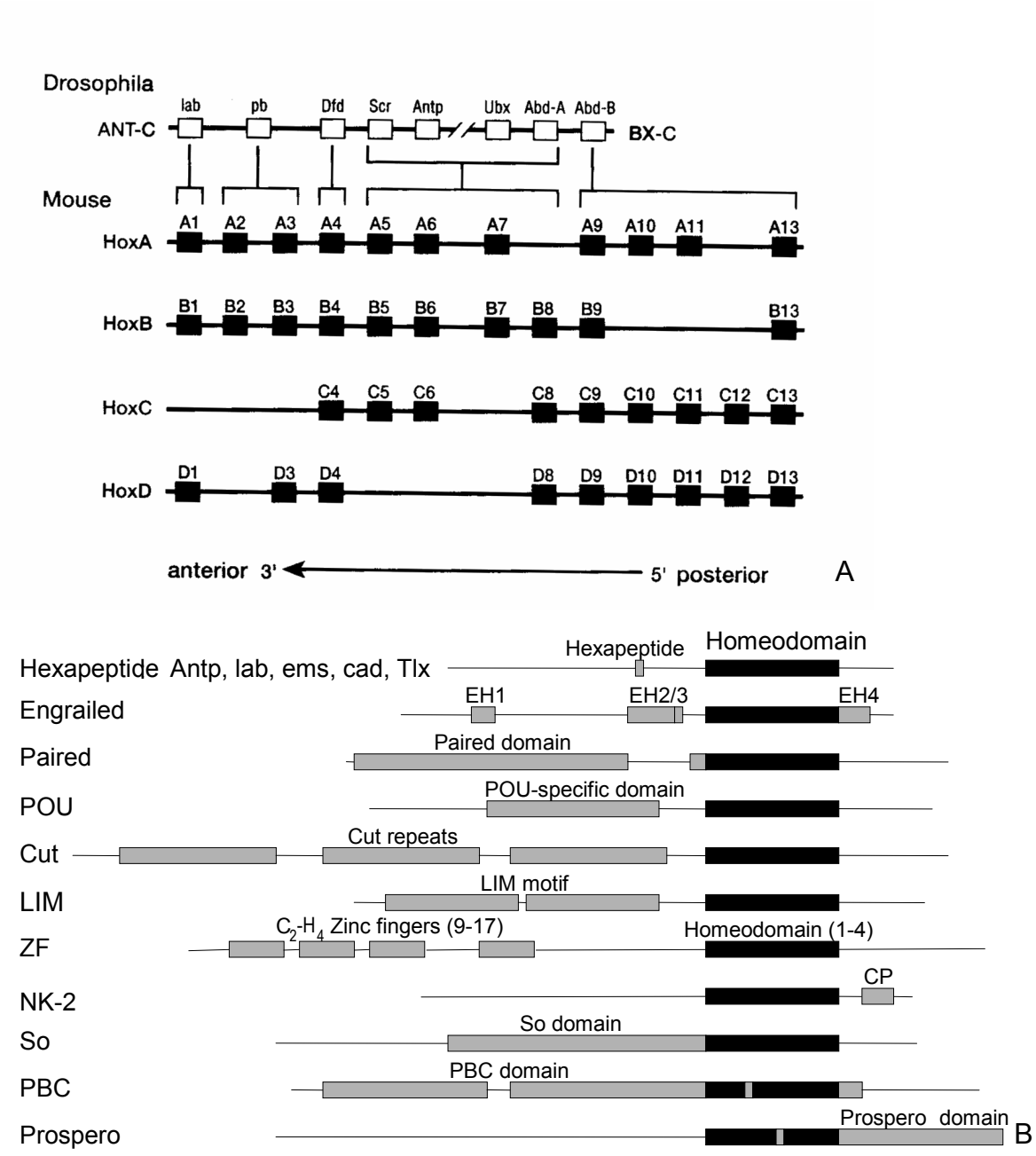


Figure 2: The homeodomain classes

(A) Alignment of the vertebrate *Hox* complex with the Drosophila *HOM-C* complex. Vertebrates have four Hox clusters, that are thought to have arisen through duplications in the genome [Kenyon, 1994]. Genes with the same numbers have shared properties and are therefore called paralogues. Solid lines denote the relationship of the *Hox* genes with the *HOM-C* genes. The polarity of transcription is indicated at the bottom. Genes at the 5' end of the complex are expressed earlier and more anteriorly than genes at the 3' end [Cappechi, 1997; De Robertis, 1994; Gehring, 1998; Krumlauf, 1994].

(B) Schematic representation of homeobox gene classes. The homeodomain is depicted in black; other conserved domains are in gray. The length of the boxes is approximately proportional to the size of the domains; the linking lines can be variable in size. Based upon Bürglin, 1994; Gehring et al, 1994.

1.4. The functions of homeobox genes in development of the nervous system

1.4.1. The early nervous system is patterned by homeobox genes

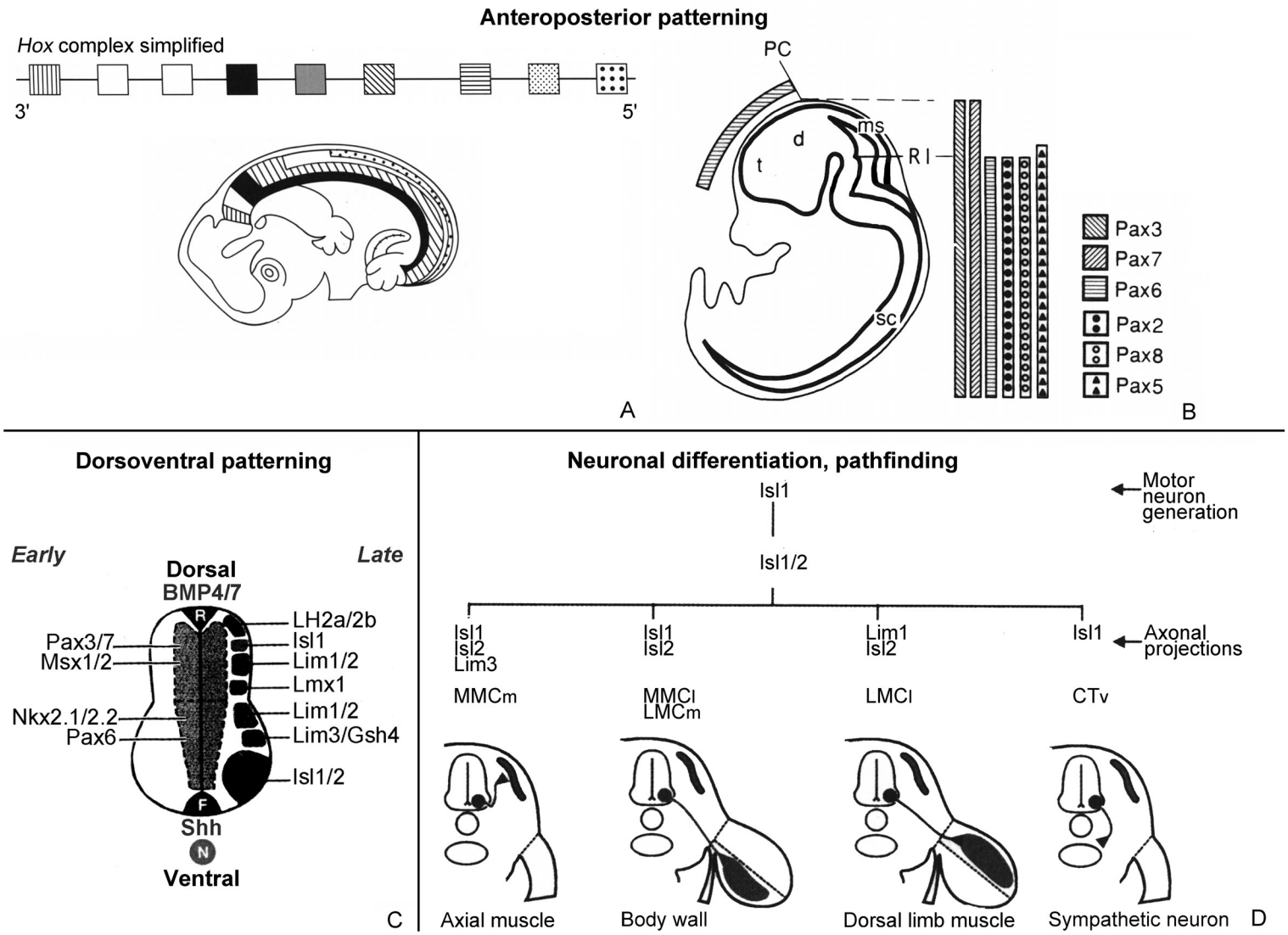
The function of the *Hox* genes in anteroposterior patterning of the nervous system is well described. *Hox* genes are induced by retinoic acid (RA) and are expressed along the axis of the embryo starting posterior with anterior expression boundaries, depending on their sensitivity to RA [Cappechi, 1997; Deschamps et al, 1999; Krumlauf, 1994]. *Hox* genes that are located more 3' in the *Hox* complex are expressed to a more anterior boundary than *Hox* genes at the 5' end, resulting in spatially restricted domains (or segments) which express different combinations of *Hox* genes (Figure 3A). A combination of *Hox* genes leads to a phenotype appropriate to that specific segment. When a *Hox* gene is lost, the combination of *Hox* genes in a segment is similar to that of a more anterior segment resulting in an anteriorization of the phenotype. A gain-of-function mutant has a posteriorized phenotype because the segment anterior to the boundary normally does not, but in the case of a gain-of-function does contain that *Hox* gene [Gehring, 1998; Jessell and Lumsden, 1997; Krumlauf, 1994]. *Pax* genes are also thought to play a role in anteroposterior patterning, because they are also expressed in a longitudinal way with anterior expression limits. Unlike *Hox* genes, which are expressed posterior to the midbrain-hindbrain border, *Pax* genes are expressed more anteriorly in the developing brain (Figure 3B) [Chalepakis et al, 1993]. The nervous system is not only patterned in an anteroposterior manner by the *Hox* and *Pax* genes, but during development also undergoes dorsoventral patterning. Cells generated from the ventral neural tube become floor plate cells, motor neurons, and ventral interneurons; cells that arise at the dorsal neural tube develop into neural crest cells, roof plate cells and dorsal sensory relay interneurons. A gradient of bone morphogenic proteins (BMPs), produced by the dorsal epidermal ectoderm, and Sonic hedgehog (Shh), secreted from the ventral notochord, results in the specification of these different cell types, marked by different homeobox genes (Figure 3C) [Jessell and Lumsden, 1997; Lee and Jessel, 1999; Shirasaki and Pfaff, 2002; Tanabe and Jessell, 1996].

1.4.2. Homeobox genes guide differentiation of neurons

At the stage of development described in the former section, signaling molecules from the cellular environment (i.e. RA, BMPs, and Shh) turn on homeobox genes, inducing the cells to become specific neuronal types, like interneurons, motor neurons, or sensory neurons. As development proceeds, homeobox gene cascades take place further promoting the differentiation of the neurons into specialized neuronal subtypes. Subclasses of spinal cord motor neurons, for example, are organized into columns. Motor neurons within such a column project to specific peripheral target muscles. These subclasses of motor neurons can be distinguished by the combinatorial expression of different LIM class homeobox genes (Figure 3D) [Shirasaki and Pfaff, 2002; Tanabe and Jessell, 1996].

1.4.3. Homeobox genes regulate outgrowth and pathfinding

Neurons that have migrated and differentiated grow out using defined pathways to find the targets they innervate. In this stage of development homeobox genes also play important roles. For example, in *Isl* mutant *Drosophila,* motor neurons exhibit pathfinding defects resulting in innervation of the wrong muscles, and fibers of interneurons fail to fasciculate. *Isl* is required not only for pathfinding in *Drosophila* but also for the neurotransmitter phenotype of neurons: interneurons that normally produce serotonin and dopamine fail to do so in the mutant [Thor and Thomas, 1997]. In *Lmx1b* mutant mice, the axons of specific motor neurons cannot sense the guidance cues that direct their axons to their appropriate targets: they randomly project into the dorsal and ventral limb. The downstream genes regulated by *Lmx1b* may be receptors recognizing guidance cues [Shirasaki and Pfaff, 2002].


Anteroposterior patterning
Hox complex simplified
3'
5'
PC
ms
d
t
R I
sc
Pax3
Pax7
Pax6
Pax2
Pax8
Pax5
A
B
Dorsoventral patterning
Early
Late
Dorsal
BMP4/7
R
Pax3/7
Msx1/2
Nkx2.1/2.2
Pax6
LH2a/2b
Isl1
Lim1/2
Lmx1
Lim1/2
Lim3/Gsh4
Isl1/2
F
Shh
N
Ventral
C
Neuronal differentiation, pathfinding
Isl1
Motor neuron generation
Isl1/2
Isl1 Isl2 Lim3
Isl1 Isl2
Lim1 Isl2
Isl1
Axonal projections
MMCm
MMCl LMCm
LMCl
CTv
Axial muscle
Body wall
Dorsal limb muscle
Sympathetic neuron
D

Previous page:
Figure 3: The functions of homeobox genes in the developing nervous system
Anteroposterior patterning of the nervous system: (A) Simplified scheme of *Hox* gene expression along the anteroposterior axis. *Hox* genes that are located more 3' in the *Hox* complex are expressed to a more anterior boundary than *Hox* genes at the 5'end [from: Jessell and Schacher, 1991]. (B) *Pax* genes are expressed more anteriorly than *Hox* genes. *Pax3*, *-5*, and *-7* are expressed in the mesencephalon; *Pax3*, *-6*, and *-7* in the diencephalon. In the telencephalon, only *Pax6* can be detected [Chalepakis et al, 1993].
d = diencephalon, ms = mesencephalon, PC = posterior commissure, RI = rhombencephalic isthmus, sc = spinal cord, t = telencephalon
Dorsoventral patterning of the nervous system: (C) Early in development, a gradient of BMPs, produced by the epidermal ectoderm, and Shh, produced by the notochord, results in the induction of dorsal and ventral cell types, marked by different homeobox genes. In the dorsal neural tube, *Pax3* and *Pax7*, *Msx1* and *Msx2* are expressed, inducing dorsal cell types. In the ventral cell types, the expression of the homeobox genes *Pax3/7*, *Msx1/2* is inhibited by Shh, whereas expression of *Nkx2.1*, *Nkx2.2*, and *Pax6* is induced. Later in development, *LIM* homeobox genes are involved in the specification of cell types along the dorsoventral axis. Developing motor neurons are dependent on the expression of the LIM homeobox gene *Isl1* and *-2*. Dorsal and ventral interneurons contain different combinations of *LIM3*, *Gsh-4*, and *LIM1/2*. Neural crest cells are marked by the zinc finger transcription factor *Slug*, dorsal commissural neurons by the LIM genes *LH2a* and *-b*. Finally, dorsal association neurons contain *Isl1* [Jessell and Lumsden, 1997; Shirasaki and Pfaff, 2002; Tanabe and Jessell, 1996]. BMP = bone morphogenic protein, F = floor plate, N = notochord, R = roofplate, Shh = sonic hedgehog.
Differentiation and pathfinding of neurons: (D) After neural induction and specification, homeobox genes are involved in differentiation of neurons. Subclasses of spinal cord motor neurons, for example, are organized into columns. Motor neurons within such a column project to specific peripheral target muscles. Motor neurons in the MMCm express *Isl1* and *-2* together with *Lim3* and innervate axial muscles close to the vertebral column. Motor neurons in the LMCl are marked by *Lim1* and *Isl2*. These neurons project their axons to limb muscles. [Shirasaki and Pfaff, 2002; Tanabe and Jessell, 1996]. CTv = preganglionic motor column of Terni, LMCl = lateral subdivision of the lateral motor column, LMCm = medial subdivision of the lateral motor column, MMCl = lateral subdivision of the medial motor column, MMCm = medial subdivision of the medial motor column

1.5. Some homeobox genes remain expressed in the adult nervous system

The role of homeobox genes in the embryonic development of the nervous system has been extensively studied, but homeobox genes are also expressed in adult neurons. In the developing nervous system, many homeobox genes are widely expressed and they undergo different patterns of subsequent restriction during maturation. For example, *Pax* genes, involved in anteroposterior and dorsoventral patterning of the neural tube and in early brain regionalization, are also expressed in adult brain structures, mainly in some brainstem nuclei and in several domains of the limbic system [Stoykova and Gruss, 1994]. In the brainstem, the thalamus and hypothalamus, POU gene expression is distributed in different combinations in different nuclei [He et al, 1989]. *Isl1*, of the LIM class, is maintained in subsets of motor and sensory neurons (see section 4.3.), and in nuclei of the brain involved in autonomic and endocrine control [Thor et al, 1991]. The function of homeobox genes in the adult is largely unknown. They are thought to play a role in the maintenance of specific neuronal phenotypes.

Summarized, homeobox genes are involved in anteroposterior and dorsoventral patterning specifying cells to become certain neuronal types. As development proceeds, homeobox genes are important for the differentiation of specific neuronal subtypes. Neuronal outgrowth and pathfinding are the next processes in which homeobox genes play important roles and finally, homeobox genes are also expressed in the adult nervous system where they are thought to play a role in maintenance of the mature neuronal phenotype.

The involvement of homeobox genes in developmental processes and their expression in the adult nervous system, has lead to the notion that in the adult organism, developmental processes may still be playing until now unknown roles. Structural plasticity, i.e. regeneration of injured nerve fibers, may be a process in which homeobox genes could be involved. In the next sections, the role of homeobox genes in the developing and adult peripheral nervous system will be described as well as the process of regeneration and the hypothesized role that homeobox genes might play therein.

2. THE PERIPHERAL NERVOUS SYSTEM: DORSAL ROOT GANGLIA

2.1. Dorsal root ganglia contain sensory neurons

The peripheral nervous system (PNS) is comprised of ganglia and peripheral nerves and provides the central nervous system (CNS), i.e. the brain and spinal cord, with information about the body and its environment. Sensory information from the skin, muscles, joints, and viscera is conveyed to the spinal cord by the sensory neurons of the PNS. The cell bodies of these neurons are located in sensory ganglia, which comprise dorsal root ganglia (DRGs) or spinal ganglia associated with peripheral nerves, and cranial sensory ganglia associated with some of the cranial nerves.

Sensory neurons in the adult DRGs are pseudo-unipolar neurons that have a central and a peripheral branch. The peripheral fiber terminates in the target tissue as a free nerve ending or innervates a specialized structure that acts as a receptor for a certain stimulus. The central fiber enters the spinal cord through the dorsal root. A stimulus from the periphery is thus conveyed to the CNS by sensory nerve fibers. The spinal cord receives sensory fibers in the dorsal horn and sends motor nerves from the ventral horn in a segmented fashion. Each spinal cord level is innervated by DRGs of the same level. The peripheral fibers of the DRG neurons together with the motor fibers of the spinal cord motor neurons form peripheral nerves that innervate skin, muscles, and joints that correspond to that spinal cord level [Augustine et al, 1997a; Larsen, 1993; Le Douarin and Kalcheim, 1999; Scott, 1992].

2.2. There are several DRG neuronal subpopulations

Several types of DRG neurons can be distinguished according to their peripheral targets, their receptors and their fiber types. Neurons innervating skeletal muscle are proprioceptive neurons that relay information about position and movement of the limbs and joints. Their peripheral nerve endings innervate mechanoceptors: muscle spindles and Golgi tendon organs. Cutaneous DRG neurons innervating skin are associated with a variety of mechanoceptors involved in the tactile sensation (touch). Other cutaneous neurons have thermoceptors detecting changes in temperature. Information from the viscera, i.e. movement of intestine, bladder wall tension and renal blood vessel pressure is detected by visceral DRG neurons with mechanoceptors. Nociceptive neurons in all target tissues described above respond to mechanical and/or thermal painful stimuli [Bergman et al, 1999; Caterina and Julius, 1999; Lawson, 1992; Martin and

Jessell, 1991a; Perl, 1992]. Touch and limb proprioception are mediated by large-diameter thickly myelinated Aα/β-fibers with a high conduction velocity. Large-diameter fibers enter the lateral aspect of the dorsal columns and ascend to the brain either directly or indirectly via second order neurons in the deeper laminae (III-IV) of the dorsal horn. Some large-diameter fibers terminate in motor nuclei (lamina IX) and mediate stretch reflexes. Slower conducting small-diameter unmyelinated C-fibers and thinly myelinated Aδ-fibers mediate sensation of pain and temperature as well as visceral stimuli. Small-diameter fibers enter the gray matter through the tract of Lissauer and terminate in the superficial layers (I and II) of the dorsal horn innervating second order neurons that ascend to the brain [Hunt et al, 1992; Martin and Jessell, 1991b].

3. DEVELOPMENT OF THE DORSAL ROOT GANGLION NEURONS

3.1. Precursors for DRGs arise from the neural crest

The embryonic nervous system starts to develop from a specialized region of the ectoderm, the neural plate. Neural crest cells are located at the lateral edges of the neural plate. During the process of neurulation the neural plate folds, leading to the formation of the neural tube. During neural tube closure, neural crest cells detach and start to migrate to several locations in the body where they form a variety of tissues and cell types (Figure 4). Neural crest derivatives include glia and neurons in dorsal root ganglia, in some cranial sensory ganglia, and in sympathetic and parasympathetic ganglia (Figure 4). Furthermore, cranial neural crest gives rise to several craniofacial bones and muscles [Bronner-Fraser, 1995; Chambers and McGonnell, 2002; Hall, 1999; Larsen, 1993; Le Douarin and Kalcheim, 1999].

The trunk neural crest gives rise to the dorsal root ganglia and to sympathetic ganglia using the ventral migration path. The first wave of neural crest cells migrates between adjacent somites, reaches the dorsal aorta and forms the primary sympathetic ganglia. The second wave of migratory neural crest cells enters the rostral half of the somites and gives rise to the DRGs and to the Schwann cells of the peripheral nerves. The segmented pathway of neural crest migration (through the rostral half of each somite) determines the segmented arrangement of DRGs, peripheral nerves and sympathetic ganglia (Figure 5A) [Le Douarin and Kalcheim, 1999].

3.2. DRG neuronal outgrowth and target innervation

3.2.1. DRG neurons grow out to innervate peripheral and central targets

In the developing DRGs neural crest cells proliferate. DRG cells reach their final mitosis (birthday) in waves: future mechanosensory cells are born early in development, peaking at E13 in the rat (about E11.5 in the mouse), whereas future nociceptive neurons are born later, peaking at E14. A third wave of neurons is generated from E15 to E16. Once born, neurons start to differentiate and to grow out to innervate their targets. Sensory axons from the DRGs form spinal nerves in a segmental fashion (Figure 5), using motor neurons that have grown out slightly earlier as guides in order to innervate the same target muscles. The first outgrowing fibers reach the hindpaw at E14, the toe region is innervated from onward E18 [Jackman and Fitzgerald, 2000; Larsen, 1993; Le Douarin and Kalcheim, 1999; Scott, 1992]. Simultaneously, DRG neurons also grow fibers that enter the spinal cord through the dorsal root, innervating interneurons in the dorsal horn and motor neurons in the ventral horn. At E14.5 mechanosensory A-fibers enter the gray matter of the spinal cord; nociceptive C-fibers appear later, at E17/18 [Jackman and Fitzgerald, 2000].

Neural crest derivatives

Neurons in:
- Cranial sensory ganglia of nerves V, VII, IX, X
- Peripheral sensory ganglia or dorsal root ganglia
- Sympathetic and parasympathetic ganglia
- Enteric plexuses

Schwann cells and satellite cells in:
- All sensory ganglia including ganglia not derived from neural crest
- Peripheral nerves
- Sympathetic and parasympathetic ganglia

Endocrine and paracrine cells
- Adrenal medulla
- Calcitonin-producing cells
- Carotid body cells

Mesectodermal structures (cranial neural crest)
- Skeleton of face and branchial arches
- Parts of the skeleton surrounding the brain
- Connective and muscular tissues of the face, eyes, arteries and glands
- Tooth papillae
- Meninges

Additional cell types
- Melanocytes

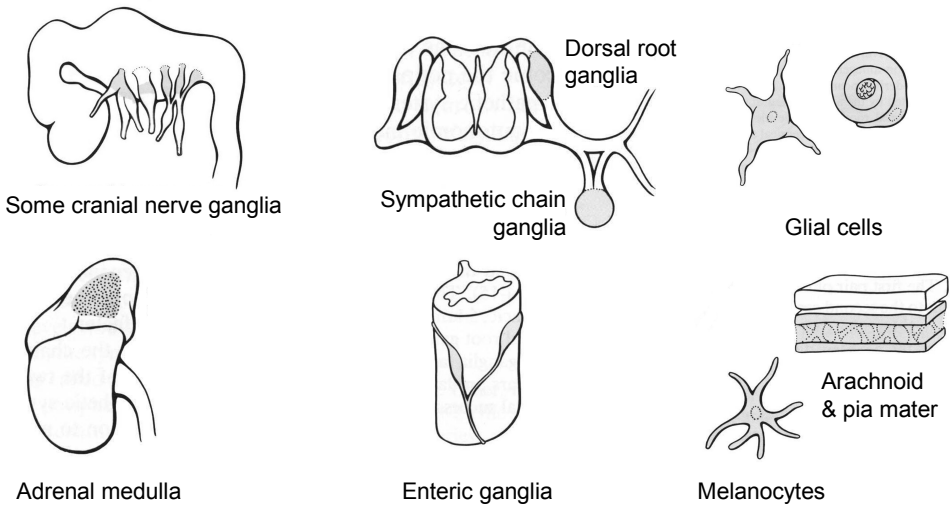


Figure 4: The major derivatives of the neural crest
Neural crest cells give rise to sensory neurons and glial cells in the ganglia of the peripheral nervous system. Several endocrine and paracrine cell types are also derived from neural crest. The cranial neural crest gives rise to many bones and tissues in the head, summarized as mesectodermal structures. The last wave of migrating neural crest cells form melanocytes and other pigment cells. Some of the (structures containing) neural crest derivatives are depicted in gray in the cartoons beneath the table. Based upon Hall, 1999; Larsen, 1993; Le Douarin and Kalcheim, 1999.

3.2.2. DRG neurons use guidance cues for pathfinding

Outgrowing neurons express B-50, also called GAP-43, a growth-associated protein that accumulates in axonal growth cones. [Jackman and Fitzgerald, 2000; Oestreicher et al, 1997; Woolf et al, 1990]. The growth cone is a specialized structure at the tip of the outgrowing axon, which functions as a sensor for axonal guidance cues: proteins in the extracellular environment (i.e. extracellular matrix, other neurites, Schwann cells and fibroblasts) that stimulate or inhibit growth, attract or repel nerve fibers. This process is called pathfinding [Augustine et al, 1997b; Dodd and Jessell, 1988; Oestreicher et al, 1997]. Examples of guidance molecules used by DRG neurons are the neurotrophins described in the next section [Bibel and Barde, 2000; Song and Poo, 1999] and cell adhesion molecules like L1 and NCAM [Augustine et al, 1997b; Davies and Lumsden, 1990; Reichardt, 1992]. Signals from guidance molecules trigger intracellular transduction pathways (for example by phosphorylation of B-50), leading to remodeling of the cytoskeleton in order to allow neurite outgrowth and changes in direction of outgrowth [Oestreicher et al, 1997].

3.3. Neurotrophic factors are essential for DRG neurons to survive

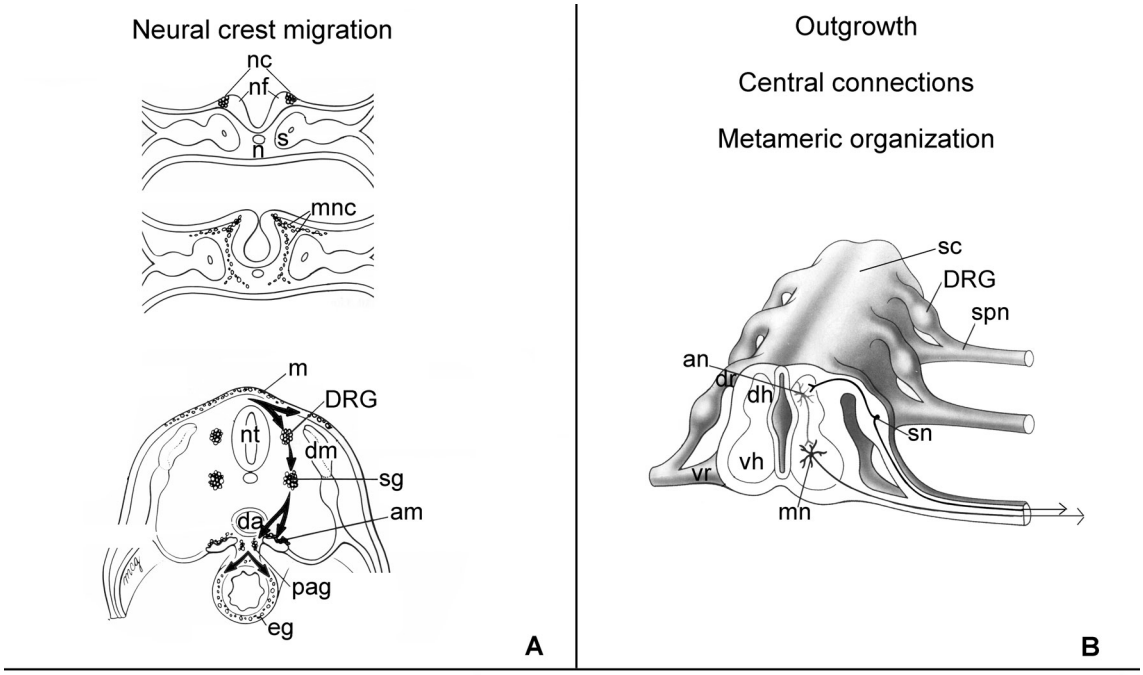
DRG neurons are generated in excess. During the period of target innervation, the sensory neurons depend on neurotrophic factors. By producing limited amounts of neurotrophic factors, the target tissues select those neurons that are able to obtain sufficient amounts of neurotrophic factors to survive. The neurons that do not get enough trophic support die by way of apoptosis [Bibel and Barde, 2000; Davies, 1992; Le Douarin and Kalcheim, 1999]. After experimental removal of a limb during early development, for example, cells migrate, proliferate and differentiate normally, but die during this period of neurotrophin dependency [Caldero et al, 1998].

The different neuronal subpopulations (see section 2.1.) express different neurotrophin receptors (Trks) and are dependent on different neurotrophins according to their function and the targets they innervate (Figure 5C). Moreover, during the period of pathfinding each neuronal subpopulation is attracted to the specific target tissue by gradients of neurotrophins. Nerve growth factor (NGF) is present in the developing skin and is needed by TrkA-positive nociceptive and thermoceptive neurons. Neurotrophin-3 (NT-3) is expressed in skeletal muscle, heart, skin, gut and liver. Muscle-derived NT-3 is essential for limb proprioceptive neurons expressing TrkC. TrkB-positive mechanoceptive neurons are supported by brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) [Bibel and Barde, 2000; Chen and Frank, 1999; Chen et al, 1999; Davies, 1994; Ernfors, 2001]. As development proceeds a population of small nociceptive neurons switches from NGF dependence in embryonic life to dependence on GDNF - glail cell line-derived neurotrophic factor - in postnatal life. These neurons express the GDNF receptors cRET and GDNF receptor-alpha [Molliver et al, 1997].

3.4. Maturation of DRG neurons

The neurons that have succesfully reached their targets survive and subsequently develop their mature phenotype. At their peripheral end, sensory receptors develop and centrally, terminal arbors and synaptic connections become mature. Peptidergic nociceptive neurons start expressing neuropeptides, like calcitonin gene-related peptide (CGRP) and substance P (SP), only when connection with the target tissue is established [Hall et al, 1997; Jackman and Fitzgerald, 2000]. The electrophysiological properties of the nerve fibers are dependent on the maturation of the Schwann cells of the peripheral nervous system that develop simultaneously with sensory neurons. Schwann cells provide trophic support for the neurons and the axons in turn produce

factors that regulate Schwann cell development [Jessen and Mirsky, 1998; Scherer, 1997]. Some neuronal subtypes, e.g. polymodal nociceptors with C-fibers, surrounded by non-myelinating Schwann cells, have fully mature membrane properties at birth, whereas myelination of A-fibers by myelinating Schwann cells occurs during early postnatal development. In the first two or three postnatal weeks all sensory neurons have obtained their mature physiological properties [Fitzgerald and Fulton, 1992. Jackman and Fitzgerald, 2000].



Target innervation and neurotrophin dependency: subpopulations of DRG neurons

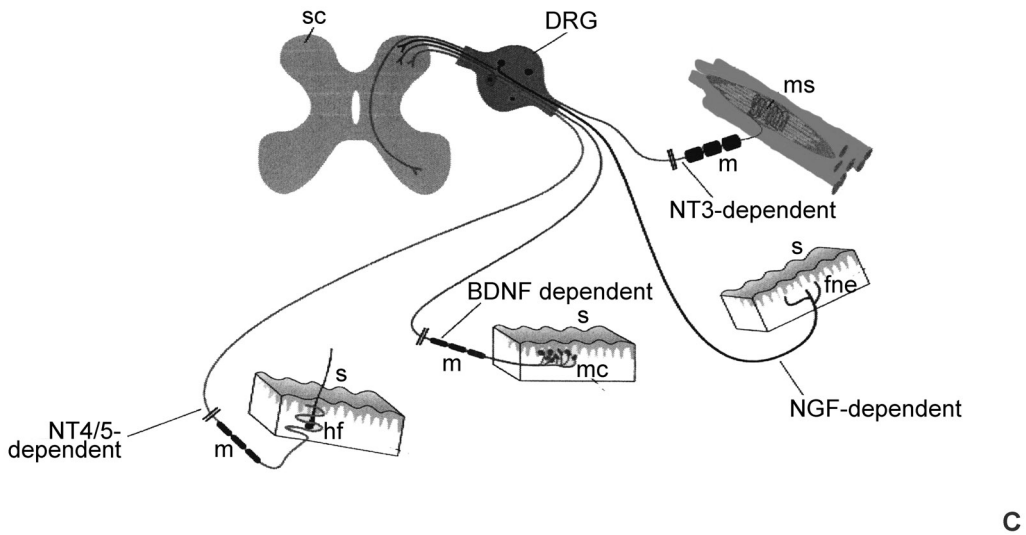


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Figure 5: DRG development: from neural crest to specific neuronal subpopulations
Upper panel: (A) Scheme of transverse sections of the early embryo at the trunk level. Neural crest cells arise at the lateral edges of the neural plate and start migrating when the neural folds close to form the neural tube. Neural crest cells that follow the ventral path of migration give rise to the enteric nervous system, the adrenal medulla, the sympathetic ganglia and the dorsal root ganglia [Larsen, 1993; Le Douarin and Kalcheim, 1999]. am = adrenal medulla, dm = dermamyotome, da = dorsal aorta, DRG = dorsal root ganglion, eg = enteric ganglia, m = melanocytes, mnc = migrating neural crest cells, n = notochord, nc = neural crest, nf = neural folds, nt = neural tube, pag = preaortic ganglia. Based upon Larsen, 1993.
(B) In the period of outgrowth, sensory neurons in the DRGs extend their fibers to their central targets, interneurons in the dorsal horn of the spinal cord, and to their peripheral targets, following the fibers from outgrowing motor neurons in the ventral spinal cord. Metameric organization: each spinal cord level is innervated by sensory neurons in DRGs of the corresponding level. an = association neuron, dh = dorsal horn, dr = dorsal root, DRG = dorsal root ganglion, mn = motorneuron, sc = spinal cord, sn = sensory neuron, spn = spinal nerve, vh = ventral horn, vr = ventral root. Based upon Larsen, 1993.
Lower panel: (C) At later stages during development outgrowing neurons get dependent on neurotrophins. Neuronal subpopulations, connecting to different sensory receptors in skin or muscle depend on different neurotrophic factors. Proprioceptive neurons, innervating muscle spindles, have thickly myelinated fibers and are dependent on NT-3. Nociceptive neurons with free nerve endings and unmyelinated fibers innervate the skin and are NGF dependent. Hair follicles and Merkel cells are innervated by thinly myelinated mechanoceptive neurons and depend on NT4/5 and BDNF respectively. BDNF = brain-derived neurotrophic factor; fne = free nerve endings, hf = hair follicle, m = myelin, mc = Merkel cell, ms = muscle spindle, NGF = nerve growth factor; NT3 = neurotrophin3; NT4/5 = neurotrophin 4/5, s = skin. Based upon Bibel and Barde, 2000.

4. HOMEOBOX GENES ARE EXPRESSED THROUGHOUT DRG DEVELOPMENT

4.1. The neural crest and its derivatives depend on homeobox genes

The role of homeobox genes in neural crest development is extensively studied in the cranial neural crest. Neural crest cells arising in the hindbrain migrate into the branchial arches from which several of the craniofacial neural crest derivatives develop (see figure 4). *Gsc*, for example, is expressed in branchial arches 1 and 2. *Gsc* knock-out mice die after birth because of craniofacial defects. *Msx1* and *-2* are expressed in the dorsal neural tube and in neural crest-derived mesenchymal tissues. They are important for tooth development and the *Msx* null mutant mouse displays defects in the head. *Pax3* and *-7* are also expressed in cephalic neural crest derivatives. [Le Douarin and Kalcheim, 1999].

Hox genes play very important roles in patterning neural crest migration in the hindbrain, which is divided into 8 segments called rhombomeres (r). *Hoxa1/Hoxb1* double mutants show a reduction in size of r4 and an inability of this rhombomere to generate neural crest cells [Gavalas et al, 2001]. In the absence of *Hoxa2*, r2 neural crest cells produce a virtually complete first branchial arch-type set of bones and mutations in *Hoxa1, -a2*, and *-a3* affect the formation of neural crest-derived cranial ganglia [Capecchi, 1997; Larsen, 1993; Le Douarin and Kalcheim, 1999].

The paired homeobox gene *Pax3* is expressed in the dorsal neural tube - including neural crest - and in adjacent somites. The *Pax3* null mutant, called *Splotch*, is defective in neural tube closure and crest cell emigration. Pigment cells, sympathetic and dorsal root ganglia, enteric neurons, and cardiac structures are absent or severely reduced in this null mutant. The defect in emigration is thought to be due to the lack of interaction between crest cells and the somites [Anderson, 1999; Le Douarin and Kalcheim, 1999; Chalepakis et al, 1993].

4.2. Homeobox genes are involved in the development of DRG neurons

Pax3 is not only important for migration of crest cells, but is also involved in the generation of DRG neurons themselves. Koblar et al (1999) showed a five-fold reduction in the ability of *Splotch* neural crest cells to generate DRG neurons *in vitro*. In cultured DRGs *Pax3* antisense oligonucleotides resulted in a 90% inhibition of production of new sensory neurons [Koblar et al, 1999].

Brn3a and *–3b*, two members of the POU class, are both widely expressed in the DRGs during development, *Brn3c* is expressed in a subset of developing DRG neurons [Akopian et al, 1996]. Knock-out studies have implicated an important role for *Brn3a* in DRG development although there are conflicting data on the severity of the phenotype. Xiang et al and McEvilly et al reported in 1996 the lack of neurotrophin receptors and the loss of specific subpopulations of sensory neurons in *Brn3a* knock-out mice. More recently Eng et al (2001) developed *Brn3a*-deficient mice expressing a LacZ reporter linked to the *Brn3a* regulatory region. They showed a disorganization of DRG axon outgrowth and extensive DRG neuronal death. *Brn3b* and *Brn3c* knock-out mice do not display any phenotype in DRG development [Gan et al, 1996; Erkman et al, 1996].

The paired-like homeobox gene *DRG11* was found in DRGs by Saito et al (1995) using RT-PCR. *DRG11* is specifically expressed in DRG neurons and in the dorsal horn of the spinal cord, where interneurons are located which receive synaptic input from the DRG [Akopian et al, 1996; Saito et al, 1995]. *DRG11* was hypothesized to play a role in specification of the neurotransmitter phenotype of DRG neurons or in synapse formation. The *DRG11* knock-out mouse, generated by Chen et al (2001), display abnormalities in innervation of the lateral dorsal horn by DRG neurons innervating the skin. Although at embryonic stages no significant DRG cell loss is found, in adult mice, almost 30% of the DRG neurons are lost. The knock-out mice display abnormalities in pain sensitivity. These data suggest that *DRG11* is required for the projections of nociceptive neurons to the spinal cord and for maintenance of DRG neurons in adult DRGs [Chen et al, 2001].

4.3. Several homeobox genes remain expressed in adult DRGs

Isl1 is expressed in adult DRGs, in a subset of neurons. Because of its expression in both neuronal and endocrine adult tissues it is thought to be involved in specification and maintenance of neural and endocrine properties of cells [Hol et al, 1999; Thor et al, 1991]. *Brn3a*, *-3b*, and *-3c* are also expressed in adult DRGs, *Brn3a* in most sensory neurons, *Brn3b* and *-3c* in small subsets of neurons [Xiang et al, 1995]. Their function in the adult DRGs is not known. In immortalized sensory cell lines *Brn3a* is involved in neurite outgrowth, induction of synaptic proteins and neurofilament genes, and plays a role in protection against apoptosis. Injection of Brn3a into neonatal sensory neurons also resulted in protection from apoptosis [Smith et al, 1997; Smith et al, 2001]. Finally, *DRG11* is expressed in a subset of adult DRG neurons and data from the *DRG11* knock-out indicate a role in maintenance of the neurons [Chen et al, 2001].

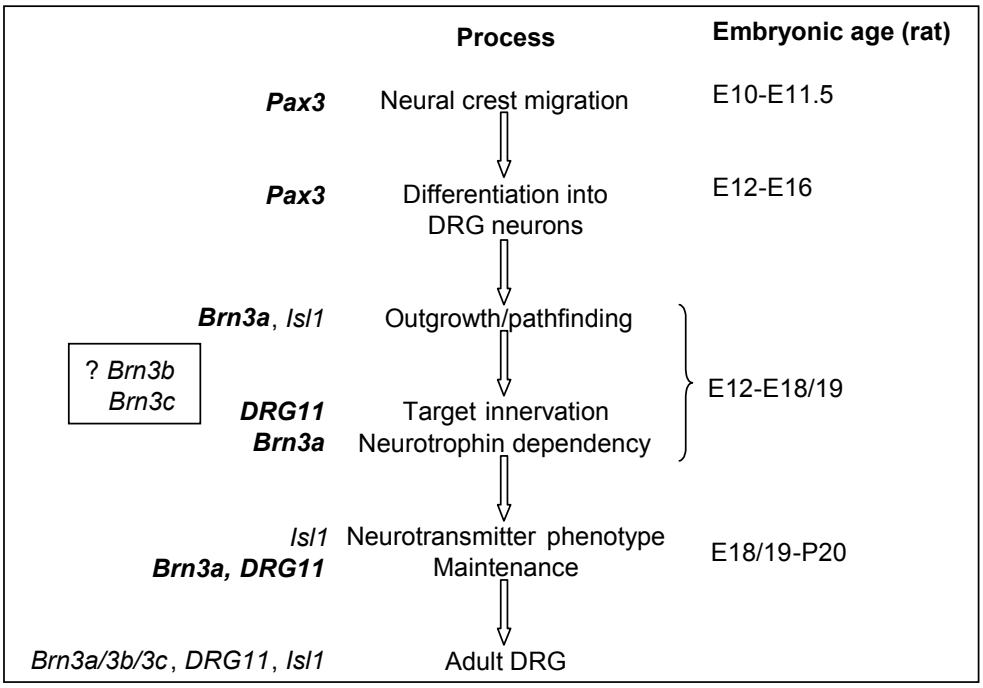

Figure 6: Expression of homeobox genes during successive stages of DRG development
Homeobox genes that have a known function in a certain process in the development of DRG neurons are typed in bold face. These include *Pax3*, involved in neural crest migration and differentiation of DRG neurons [Koblar et al, 1999]; *Brn3a*, essential for neurite outgrowth, expression of neurotrophin receptors, and neuronal survival (maintenance) [Eng et al, 2001]; and *DRG11*, involved in central target innervation and neuronal maintenance [Chen et al, 2001]. The functions of *Isl1* in DRG development are largely unknown, but in other types of neurons it is involved in outgrowth and neurotransmitter phenotype [Hol et al, 1999; Thor et al, 1991]. *Brn3b* and *-3c* are expressed in developing DRG, but their functions in DRG development are unclear [Erkman et al, 1996; Gan et al, 1996]. Finally, adult DRG neurons express *Brn3a*, *-3b*, *-3c*, *DRG11*, and *Isl1*, but their functions in the adult DRG are unknown. See for more detail sections 4.2. and 4.3.

In general, the function of homeobox genes in adult DRGs is largely unclear. In the next sections, a speculative role for homeobox genes in regeneration of the peripheral nervous system will be introduced.

5. THE PERIPHERAL NERVOUS SYSTEM IS CAPABLE OF REGENERATION

In general, the mammalian nervous system is limited in its capacity to regenerate. Once differentiated, a neuron is too specialized to proliferate, so that there is little or no self-renewal of neurons. Some neuronal structures, such as the hippocampus and the nasal epithelium, contain stem cells that are able to give rise to new neurons, but in general, if there is an excess of neural cell death, the nervous system is highly disturbed. After damage to the axon of the neuron, therefore, the neuron has to survive and regenerate its fiber to reinnervate its target. In the CNS, neurons are hardly able to regenerate their fibers mainly because of the formation of scar tissue providing an inhibitory environment for outgrowth [De Winter et al, 2002; Fawcett et al, 2002; Küry et al, 2001b; Stoll et al, 2002].

Neurons of the peripheral nervous system, in contrast, are able to regenerate their peripheral axons after injury, allowing complete functional recovery. The sciatic nerve, innervating the hindpaw of the animal, is frequently used to study peripheral nerve regeneration. After crush injury, the fibers distal to the lesion undergo Wallerian degeneration: the axon and myelin degenerate and are ingested by Schwann cells and invading macrophages. Schwann cells surrounding the distal fibers proliferate so that the endoneurial tubes surrounding the original nerve fibers remain intact, providing the environment through which the regenerating axons can grow (Figure 7). Axons begin to regenerate within a few hours after the crush lesion. Fibers in the proximal nerve stump start to sprout and new axons extend through the distal endoneurial tube to reinnervate their target organs [Allt, 1976; Fawcett and Keynes, 1990; Fu and Gordon, 1997; Stoll et al, 2002]. In the course of regeneration Schwann cells remyelinate, a process that is dependent on contact with regrowing axons. Finally, functional recovery has been shown monitoring the return of sensory and motor function [Bridge et al, 1994; de Koning and Gispen, 1987]

5.1. DRG neurons respond to injury by changing their expression profile

Peripheral nerve regeneration is accompanied by a variety of changes in the cell bodies of the DRG neurons: anatomical and morphological changes as well as changes in gene expression and cellular metabolism. The main morphological reaction of the cell body is “chromatolysis”, the disintegration of rough endoplasmic reticulum, normally packed in large granular condensations. Furthermore, swelling of the cell body has been observed and the nucleus tends to move to the periphery of the cell body. Nuclear volume increases and nucleoli enlarge indicating that the response to injury is highly anabolic [Fawcett and Keynes, 1990; Lieberman, 1971].

Regeneration is associated with the expression of new genes and proteins [Aldskogius, 1992; Fawcett and Keynes, 1990; Fu and Gordon, 1997] (Figure 7). In general, transcription and translation of many proteins involved in neurite outgrowth during embryonic development are upregulated after nerve injury. The synthesis and axonal transport of cytoskeletal proteins such as actin, tubulin, and peripherin are induced by axon injury [Aldskogius, 1992; Fawcett and Keynes, 1990], as well as the growth-associated protein B-50 [Oestreicher et al, 1997; Plantinga et al, 1993a; van der Zee et al, 1989; Woolf et al, 1990]. The upregulation of neuropilin1 (the semaphorin receptor) in DRG neurons and netrin-1 in Schwann cells suggest a role for developmental guidance molecules in regeneration [Gavazzi et al, 2000; Madison et al, 2000]. Neurotrophin production by Schwann cells is also upregulated after nerve crush, presumably involved in stimulation of neurite outgrowth and neuronal survival [Fawcett and Keynes, 1990; Fu and Gordon, 1997; Küry et al, 2001b; Lindsay et al, 1992; Verge et al, 1996]. On the other hand, proteins that have in the adult organism a function in neurotransmitter release or more general, in the adult phenotype of the neurons, are downregulated. Examples are the neurotransmitters SP and CGRP, and neurofilament proteins, that are normally expressed only late in development, when the neuron has reached its target [Aldskogius et al, 1992; Fawcett and Keynes, 1990; Hökfelt et al, 1994].

The observation that genes expressed during embryonic DRG development are upregulated and genes involved in mature neuronal function are downregulated has lead to the hypothesis that regeneration may be a recapitulation of developmental programs [Fawcett and Keynes, 1990; Skene, 1989; Wong and Oblinger, 1990].

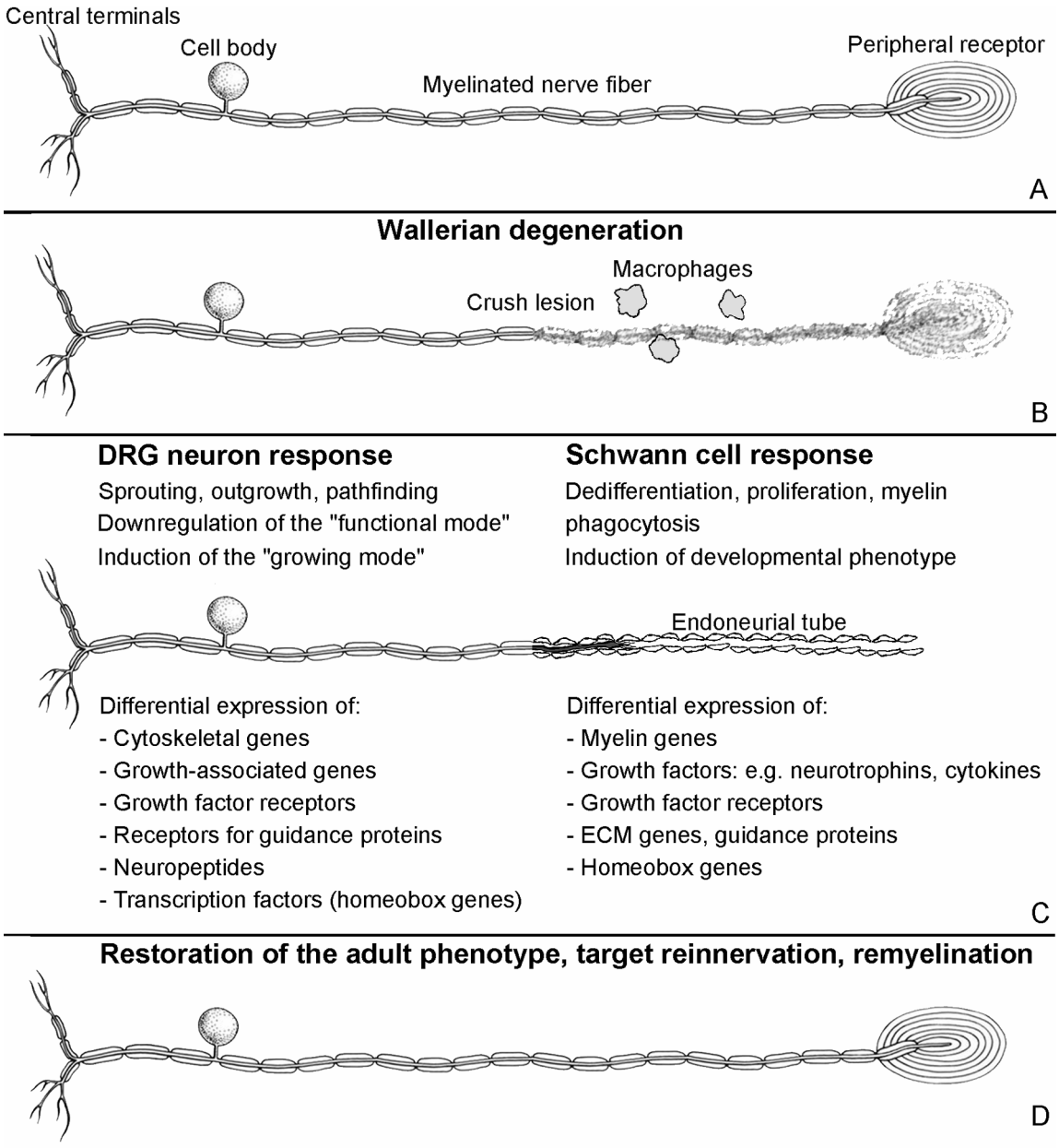

Figure 7: Schematic picture of the regenerative processes after sciatic nerve crush
A neuron with myelinated fibers is drawn as a model, but the changes described occur either in all or in certain neuronal subpopulations. (A) The adult system without a lesion. DRG neurons are pseudo-unipolar neurons with terminals in the spinal cord (central) and sensory receptors in the skin and muscles (peripheral). Picture from Martin and Jessell, 1991a (B) After a crush lesion, the peripheral fibers degenerate and Schwann cells demyelinate. Myelin and axon debris are removed by macrophages. This process is called Wallerian degeneration [Bridge et al, 1994; Fawcett and Keynes, 1990] (C) Schwann cells proliferate so that the endoneurial tube surrounding the original fibers remains intact. The proximal nerve stump starts to sprout and extend new fibers. Changes in gene expression occur such that genes associated with the function of the neurons are downregulated, whereas genes associated with outgrowth and pathfinding are upregulated [Aldskogius, 1992; Fawcett and Keynes, 1990; Fu and Gordon, 1997; Hökfelt et al, 1994]. Schwann cells upregulate neurotrophic factors and guidance molecules in order to support neuronal survival and pathfinding. Myelin genes are downregulated by the Schwann cells [Küry et al, 2001b]. (D) Regeneration leads to reinnervation of the target tissue and remyelination of the nerve fibers. The adult phenotype of the neurons is restored with respect to gene expression and function.

5.2. Homeobox genes may play a role in regeneration

As described above, in response to nerve injury, there are numerous changes in gene expression in DRG neurons, enabling the cells to downregulate their "functional mode" and to induce their "growing mode". Regeneration-associated changes in gene expression are likely to be mediated by changes in the levels of transcription factors. Much research has been done on the involvement of homeobox genes in the Schwann cell after nerve crush. It is generally proven that Schwann cells repeat their developmental program after nerve injury as was shown by many studies on gene expression, including studies on homeodomain proteins. Schwann cells in the distal stump re-express the POU class homeobox gene *Oct6/SCIP* [Gondré et al, 1998; Sherer et al, 1994] and the paired class gene *Pax3* [Küry et al, 2001a]. *Pax3* is known to be involved in the embryonic differentiation of Schwann cells from neural crest cells, and is expressed in non-myelinating Schwann cells during development. *Oct6/SCIP* is a marker for Schwann cell precursors in the promyelinating stage: its upregulation again indicates a reinduction of the developmental Schwann cell phenotype. Members of the *Hox* complex, *Hoxb5*, *Hoxd3*, and *Hoxa6*, were reported to decline in Schwann cells after nerve crush [Küry et al, 2001a, and references therein]. In DRG neurons, Hol et al showed in 1999 a down-regulation of *Isl1*. An upregulation of *Oct2* was observed by Begbie et al (1996).

5.3. Gene expression changes do not always recapitulate development

Although at first sight, gene expression alterations in injured neurons seem to reflect developmental gene expression, when examined in more detail this is not always the case. In general, tubulin is upregulated, however, some of the developmentally expressed tubulin subclasses are not [Moskowitz et al, 1993]. In line with this, the adult rather than the embryonic pattern of microtubule-associated proteins (MAPs) is retained [Fawcett et al, 1994, Ma et al, 2000]. Schwann cells upregulate L1 and NCAM [Tacke and Martini, 1993], but DRG neurons do not, inducing close homologue L1 (CHL1) instead, which is expressed predominantly during later stages of development [Zhang et al, 2000]. These findings indicate that genes involved in outgrowth and pathfinding during development are not always involved in these processes during regeneration. Similarly, the involvement of neurotrophins seems not to fully recapitulate development. Neurotrophins are known to be important attractants and survival factors for DRG neurons during development, and exogenous application of neurotrophins during regeneration have beneficial effects [Lewin et al, 1997; Mohiuddin et al, 1999; Raivich and Kreutzberg, 1993]. However, their high affinity (Trk) receptors are not upregulated after injury; TrkA is even downregulated [Mohiuddin et al, 1999; Raivich and Kreutzberg, 1993; Verge et al, 1989]. Many other (neurotrophic) growth factors are also involved in regeneration, like GDNF [Bennett et al, 1998], neuroactive cytokines [reviewed in Murphy et al, 1997; Unsicker et al, 1992], fibroblast growth factor (FGF) [Grothe et al, 2001; Grothe and Nikkhah, 2001; Ji et al, 1995], insulin-like growth factor (IGF) [Craner et al, 2002; Pu et al, 1995; Raivitch and Kreutzberg, 1993], and transforming growth factor (TGF) [Xian and Zhou, 1999], some of which have known roles in DRG development, others do not. Moreover, there are now many examples of genes not (reported to be) expressed in developing DRGs, which are upregulated after injury, e.g. FGR receptor 3 (FGFR3) [Grothe et al, 2001; Oellig et al, 1995], vasoactive intestinal protein (VIP) and neuropeptide Y (NPY) [Bergman et al, 1999; Hökfelt et al, 1994; Jazin et al, 1993; Villar et al, 1989], and small proline-rich repeat protein 1A (SPRR1A) [Bonilla et al, 2002]. Finally, some genes are induced in large neurons, whereas they are involved in development of small DRG neurons. Examples of these include neuropilin1 [Gavazzi et al, 2000], peripherin [Larivière et al, 2002; Wong and Oblinger, 1990], SP [Noguchi et al, 1994], and galanin [Holmes et al, 2000; Villar et al, 1989].

6. AIM OF THE THESIS

We are interested in the molecular mechanisms underlying regeneration-associated alterations in gene expression of sensory neurons. As described above many studies on alterations in gene expression after nerve crush suggest a reinduction of the developmental mechanisms in regenerating DRG neurons. However, there are also many reports that contradict the developmental recapitulation. The aim of this thesis is to answer the question whether the molecular mechanisms of DRG neuron regeneration recapitulate developmental mechanisms. We chose for homeobox genes as a tool since they have a fundamental role in regulating gene expression during DRG development. Based upon the well-described recapitulation of development in Schwann cells after injury, and the involvement of homeobox genes therein, we hypothesize that, if developmental mechanisms are utilized by regenerating DRG neurons, homeobox genes are likely to be involved. We expect that homeobox genes that are expressed in DRG neurons during the time period of axonal outgrowth, pathfinding, and target innervation would be reexpressed or upregulated after sciatic nerve crush. Homeobox genes expressed at later time points or involved in other processes like determination of neurotransmitter identity or maintenance of the adult phenotype should be downregulated after injury.

7. MODEL AND TOOLS

7.1. Model: sciatic nerve crush versus sciatic nerve transection

To study regeneration of the sciatic nerve several types of lesions can be used, with different outcomes of regeneration. The crush lesion of the sciatic nerve allows full recovery of target innervation and nerve function [de Koning et al, 1986; Bridge et al, 1994]; accompanying gene expression changes generally return to normal when regeneration has been achieved. The second lesion model used extensively is the complete transection of the sciatic nerve. Regenerating fibers have to traverse a gap and find the endoneurial tubes in the distal nerve stump. Regeneration is less complete, and often accompanied by neuronal cell death [Baranowski et al, 1993; Schmalbruch, 1987]. In general, gene expression changes occur after transection similar as after crush, sometimes more pronounced, but often the expression profile does not return to normal levels after transection. The transection model, therefore, is often used, sometimes in combination with ligation of the nerve stump, as a model allowing no regeneration. For these reasons, we have chosen the sciatic nerve crush model to study regeneration. Although there are only a few reports on cell death after crush, in general it is much less extensive than after transection [Baranowski et al, 1993; Fu and Gordon, 1997]. Moreover, McKay Hart et al (2002) reported that neuronal death following nerve transection was prevented by surgical nerve repair in rats, indicating that a paradigm allowing regeneration (e.g. nerve crush) would prevent neuronal death.

7.2. Tissue: sciatic nerve versus DRG

The type of assay and the tissue used to analyze the changes in gene expression greatlydetermines the type of data that is obtained. Studies on mRNA expression in sciatic nerve provide information about the gene expression in Schwann cells, fibroblasts, or endothelial cells, constituting the endoneurial tubes and of macrophages that are activated upon nerve injury. Although there are reports on local mRNA in DRG axons *in vitro*, this has not yet been shown *in vivo*. Moreover, only cytoskeletal proteins have been shown to be translated in the regenerating axon *in vitro* [Zheng et al, 2001].

As we are interested in the molecular mechanisms underlying regeneration-associated alterations in gene expression of sensory neurons, we used the DRG itself for analysis. The DRG also contains Schwann cells, but regeneration-associated changes in Schwann cells mainly occur in the distal nerve stump. Some changes in gene expression are reported in satellite cells surrounding the sensory nerve cell bodies, but most of the gene expression changes observed in DRGs occur in the sensory neurons themselves. A drawback of using whole DRG homogenates is that there are many subfamilies of sensory neurons, each with their own combination of markers and with their own functions [Lawson, 1992]. Some regeneration-associated changes in gene expression are subpopulation-specific and, therefore, may be diluted by using the whole DRG. However, in general, the changes in gene expression in subpopulations of neurons can be detected by RT-PCR or Northern blotting, and localized by *in situ* hybridization. There have been only few reports on genes upregulated in one, and downregulated in another neuronal subtype [Gavazzi et al, 2000; Noguchi et al, 1994]. In view of the aim of the study, we want to focus on those responses to injury that are likely to be similar in all neuronal subpopulations, because they all have to adopt a "growing mode" and downregulate their "functional mode" [Fawcett and Keynes, 1990].

8. OUTLINE

The studies were initiated by a screen for homeobox genes expressed in adult dorsal root ganglia of the rat (**chapter 2**) in order to identify those homeobox genes that could be used as tools in the follow-up experiments and to obtain a full picture of the uninjured state of DRG neurons. Based upon a similar screen for regeneration-associated changes in homeobox gene expression, it was decided to specifically quantify a selected number of homeobox genes in the course of regeneration. To quantify the candidate genes we switched to the mouse, in order to have the opportunity to extend studies toward specific functions of homeobox genes due to the availability of knock-out mice. **Chapter 3** describes a functional characterization of sciatic nerve regeneration in the C57BL/6J mouse strain. This strain was used for real-time quantitative PCR on a selected number of homeobox genes in **chapter 4**. The putative involvement of *Gsc* in development of the DRGs was examined in *Gsc* null mutant mice in **chapter 5**. Finally, in **chapter 6**, the data obtained are discussed in relation to the hypothesis that the molecular mechanisms of regeneration may be a recapitulation of developmental processes.

Chapter 2

Homeobox gene repertoire in adult rat dorsal root ganglia

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Neuroscience Research Communications, *in press*

ABSTRACT

Homeobox genes encode transcription factors that play key roles in embryonic development of the nervous system. Dorsal root ganglia are part of the peripheral nervous system that arises from the neural crest. Several homeobox genes are known to play important roles in the development of neural crest and dorsal root ganglia. Some of these remain expressed in the adult dorsal root ganglia (DRGs). In order to get more insight into the homeobox gene repertoire in adult rat DRGs, we performed RT-PCR with degenerate primers and identified twenty-two homeobox genes. We found homeobox genes that were reported before in embryonic or adult DRGs, as well as homeobox genes associated before with neural crest (derivatives). Some of the genes were not reported in relation to neural crest or DRGs before. Two homeobox genes displayed sequence differences with their mouse counterparts, possibly being close homologues. The diversity of homeobox genes expressed in adult DRGs suggests that gene regulatory events initiated during development remain operative in the mature DRG.

INTRODUCTION

Homeodomain (HD) proteins, encoded by homeobox genes, are transcription factors that exert their DNA binding function via the highly conserved homeodomain. Several HD protein families - like the paired, LIM, and POU families - are distinguished by the presence of additional domains, which further specify their DNA binding properties and their interaction with other proteins [Bürglin, 1994; De Robertis, 1994; Gehring et al, 1994]. During embryonic development of the nervous system HD proteins are important for anteroposterior and dorsoventral patterning [Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996] and play key roles in migration, outgrowth, differentiation, and maintenance of neurons [Akopian et al, 1996; Chalepakis et al, 1993; Hobert and Westphal, 2000].

The peripheral nervous system originates from the neural crest, which forms at the lateral ridges of the closing neuroepithelium. As the neural tube closes neural crest cells detach and follow defined migration routes, finally reaching target embryonic sites where they settle and differentiate [Le Douarin and Kalcheim, 1999]. Depending on their rostrocaudal position, neural crest cells form a variety of tissues: from craniofacial mesectodermal structures to neurons and glia in sensory and autonomic ganglia [Le Douarin and Kalcheim, 1999]. Homeobox gene expression in the neural crest and its derivatives, especially in the cranial part, has been extensively studied. *Hoxa1* and *Hoxa2* are thought to be important in patterning the neural crest at rhombomeric levels. *Pax3*, *Gsc*, *Dlx1-7*, *Otx1*, and *Msx1* are, among others, essential for the development of craniofacial derivatives of the neural crest [Le Douarin and Kalcheim, 1999].

Dorsal root ganglia (DRGs) contain the neurons that convey sensory information from the periphery to the central nervous system. The sensory neurons as well as the supporting satellite and Schwann cells are all derivatives of the neural crest [Le Douarin and Kalcheim, 1999]. Embryonic DRG neurons express the POU homeobox genes *Brn3a* and *Brn3b* [Akopian et al, 1996; He et al, 1989; Xiang et al, 1995], the paired/paired-like genes *DRG11*, *Pax3* [Saito et al, 1995], *Prx3* [Semina et al, 1998], and *Ptx2* (D.B. Jacoby, personal communication), and the LIM homeobox genes *Isl1* and *-2* [Akopian et al, 1996]. *Brn3a* is essential for outgrowth and survival of sensory neurons during DRG development [Eng et al, 2001]. *DRG11* is required for the formation of projections from nociceptive neurons to their central targets [Chen et al, 2001].

In general, the role of homeobox genes in embryonic development of the nervous system has been widely investigated. Although the expression of homeobox genes in the adult animal has been studied less intensively, evidence emerges that several of these remain expressed in the adult nervous system [He et al, 1989; Stoykova and Gruss, 1994]. With respect to the DRGs, it has been described that *Brn3a* and *Brn3b* [He et al,1989; Xiang et al, 1995], *Isl1* [Hol et al, 1999], and *DRG11* [Saito et al, 1995] are still expressed in adult ganglia. In this study, a systematic characterization of the homeobox gene repertoire in adult DRGs was performed using a PCR-based strategy.

MATERIALS AND METHODS

Experimental animals and surgery

Eight young adult male Wistar rats (Wistar: Unilever, Central Animal Faculty, Utrecht University, the Netherlands), weighing 120-140 g were killed by decapitation. From six rats, the DRGs from level L4-L6, corresponding to the sciatic nerve, were quickly dissected, cleaned from surrounding tissue, pooled and frozen on dry ice for RNA isolation. From two rats the L5 DRGs were embedded in TissueTek and frozen on dry ice for *in situ* hybridization. The animal procedures were performed in accordance with the Ethical Committee on Animal Experiments of the University Medical Center Utrecht.

RT-PCR and cloning

Total RNA (DNase-treated) from the pooled L4-L6 DRGs was subjected to RT-PCR with degenerate primers. The HD PCR primers were located within the homeobox (Figure 1) [Asbreuk et al, 2002a; van Schaick et al, 1997]: forward 5'-GMRSCGMSAVMGSACMMBCTTYAC-3' and reverse 5'-CAYYTKGYGCDRCGRTTBYKRAACCA-3'. The forward primer of the POU PCR was located in the POU-specific domain, the downstream primer in the homeobox (Figure 1): forward 5'-AASAACATGWKYRMDYTVAARCC-3' and reverse 5'-TGBCKBYKRTTRC-ARAACCARAC-3'. The annealing temperatures were 45-50 ºC. The PCR products of the appropriate lengths - 160 bp and 265 bp respectively - were ligated into pCR-Script SK(+) and transformed into E. Coli XL10Gold ultra competent cells (Stratagene) according to the manufacturer's recommendations. The inserts were identified by sequencing, according to the Beckman CEQ2000 Dye Terminator Cycle Sequencing Protocol.

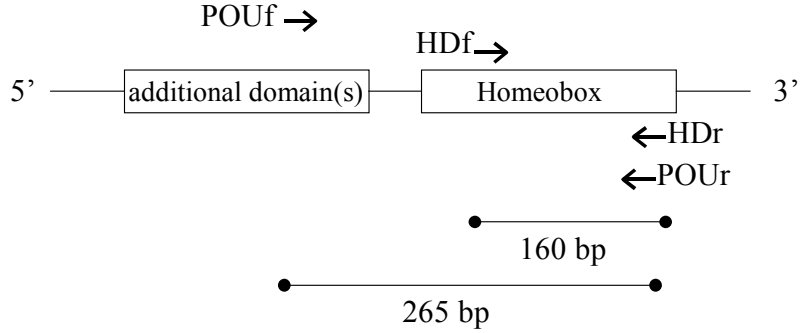

Figure 1:
Schematic representation of homeobox gene structure and position of the primers
Each homeobox gene contains a homeobox, which encodes the DNA binding homeodomain. In addition, several homeodomain protein families contain other domains, like the POU-specific domain (POU family), the paired domain (paired family), and the LIM domains (LIM family). The HD and POU PCR products are 160 and 265 basepairs (bp) in length. f = forward, r = reverse

RNA probes

Digoxygenin-labeled RNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Antisense and sense probes were used encoding *Brn2**, *Brn3a* and *–3b* [Theil et al, 1994], *Brn4**, *DRG11**, *Gbx2* [Bulfone et al, 1993], *Gsc**, *Gsh4* [Li et al, 1994], *Hoxa1* (Urrutia, U93092), *Hoxc5* [Gaunt et al, 1990], *Lmx1b* [Chen et al, 1998], *Oct1* (Hauschka, RNU17013), *Oct6* [Zwart et al, 1996], *Otp* [Simeone et al, 1994], *Pax3* (Goulding, NM_008781), *Prx2* [Opstelten et al, 1991], *Prx3* [van Schaick et al, 1997], *Ptx2**, and *Zfh4* [Kostich and Sanes, 1995]. The probe length varied from about 200 to 1300 bp. Probes marked with an asterisk are obtained from subcloned PCR products or cDNAs available at our own facilities.

In situ hybridization

In situ hybridization was carried out as described by Schaeren-Wiemers and Gerfin-Moser (1993), with minor changes. In brief, cryostat sections of the DRGs, cut at 8 µm thickness, were thaw-mounted onto SuperFrost®Plus slides (Menzel-Glaser), dried and fixed for 10 min in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS sections were acetylated for 10 min in a solution containing 245 ml H_2O, 3.3 ml triethanolamine, 438 µl HCl (37%) and 625 µl acetic anhydride. Sections were then washed with PBS and prehybridized for 2 h at room temperature in a hybridization solution (50% deionidized formamide, 5*SSC, 5*Denhardt's solution, 250 µg/ml baker's yeast RNA and 500 µg/ml herring sperm DNA). Hybridization was performed overnight at 72°C with 300-1300 ng/ml digoxygenin-labeled RNA probe in 150 µl hybridization buffer, covered with Nescofilm. The Nescofilm strips were removed by soaking in 5*SSC at 72°C and, following washing for 2 h at 72°C in 0.2*SSC, the slides were transferred to 0.2*SSC at room temperature and washed for 5 min at room temperature with buffer 1 (100 mM Tris HCl, pH7.4; 150 mM NaCl). Preincubation with 1.5 ml buffer 1 with 10% heat inactivated fetal calf serum (hiFCS) was performed for 1 hour at room temperature in a humidified chamber. The sections were then incubated overnight at 4°C with alkaline phosphatase-conjugated mouse anti-digoxygenin Fab fragment (Boehringer Mannheim), 1:5000 diluted in buffer 1 with 1% hiFCS. Following washing with buffer 1 and equilibration with buffer 2 (100 mM Tris HCl, pH9.5; 50 mM $MgCl_2$; 100 mM NaCl), the color reaction was performed in the dark for 24 h at room temperature with 200 µl NBT/BCIP solution (Boehringer Mannheim) and 2.4 mg levamisole in buffer 2. The slides were then washed with TE buffer and dH_2O, dehydrated and coverslipped with Entellan.

RESULTS

Homeobox gene repertoire of adult rat DRGs

A systematic characterization of the homeobox gene repertoire in adult DRGs was performed using a degenerate RT-PCR strategy to amplify expressed homeobox mRNAs in DRGs from young adult rats. Two primer sets were used (Figure 1). The first set of primers was positioned within the highly conserved homeobox and was originally designed to amplify members of the paired and paired-like family of homeobox genes [Smidt et al 1997]. This PCR will be referred to as the HD PCR.

The second set of primers was designed to amplify the POU family of homeobox genes, the forward primer being positioned within the POU specific domain and the reverse primer in the POU homeodomain. This second PCR will be referred to as the POU PCR (Figure 1).

To check for genomic DNA contamination, we performed control experiments adding RNA instead of cDNA to the PCRs. These PCRs did not result in a product, indicating that there was no DNA contamination (data not shown).

The HD PCR on DRGs from adult rats resulted in a DNA band of 160 basepairs (bp) that was subcloned. Clones were sequenced and the inserts were identified resulting in 16 different homeobox genes (Table 1). Ten of these clones were 100% identical to rat *DRG11*, *Hoxa1*, *Hoxc5*, *Gsh4*, *Msx1*, *Otp*, *Prx2*, *Prx3*, *Ptx2*, and *Zfh4* sequences in the Genbank nucleotide database. Four clones, *Gbx2*, *Gsc*, *Lmx1b*, and *Pax3* were most similar to mouse sequences. For these genes no rat sequences are available in the Genbank, but the translated amino acid sequences of the rat fragments were identical to the amino acid sequences of their mouse homologues. To two more clones, no definitive homologues could be assigned. The rat *Msx3-like* had 6 nucleotide differences with the mouse *Msx3* sequence in the database, 2 of which resulting in a leucine to serine transition at position 26 of the homeodomain. *Vsx2-like* had 3 different nucleotides as compared to mouse *Vsx2* (*Chx10*) yielding an isoleucine instead of a leucine at position 40 (Figure 2).

Table 1: The homeobox genes expressed in adult rat DRGs

nr	Homeobox gene	Homeobox class	Genbank accession nr.
	HD PCR		
1	*DRG11*	paired-like	U29174
2	*Gbx2*	GBX class	AF390072*
3	*Gsc*	paired-like	AY169318*
4	*Gsh4*	LIM	S71659
5	*Hoxa1*	Hox complex	U93092
6	*Hoxc5*	Hox complex	U28071
7	*Lmx1b*	LIM	AF390073*
8	*Msx1*	Msx family	D83036
9	*Msx3-like*	Msx family	AF390078*
10	*Otp*	paired-like	J10413
11	*Pax3*	paired	AF390074*
12	*Prx2*	paired-like	X52875
13	*Prx3*	paired-like	AJ002258
14	*Ptx2*	paired-like	AF039832
15	*Vsx2-like*	paired-like	AF390079*
16	*Zfh4*	zinc finger-HD	L36173
	POU PCR		
17	*Brn2*	POU	L27663
18	*Brn3a*	POU	AF390075*
19	*Brn3b*	POU	AF390076*
20	*Brn4/RHS2*	POU	Z11834
21	*Oct1*	POU	U17013
22	*Oct6*	POU	NM_011141

The homeobox genes identified using RT-PCR on mRNA from DRG L4-L6. The homeobox gene classes to which they belong and the Genbank accession numbers are shown in the right columns. Accession numbers marked with asterisks represent newly submitted sequences.

The POU PCR resulted in a band of 265 bp containing 6 different POU-homeobox genes (Table 1). Four of these clones were identical to rat *Brn2*, *Brn4*, *Oct1*, and *Oct6* Genbank database sequences. Two clones, *Brn3a* and *Brn3b* differed at 2 and 8 nucleotide positions respectively as compared to their mouse homologues, with no rat-mouse amino acid differences.

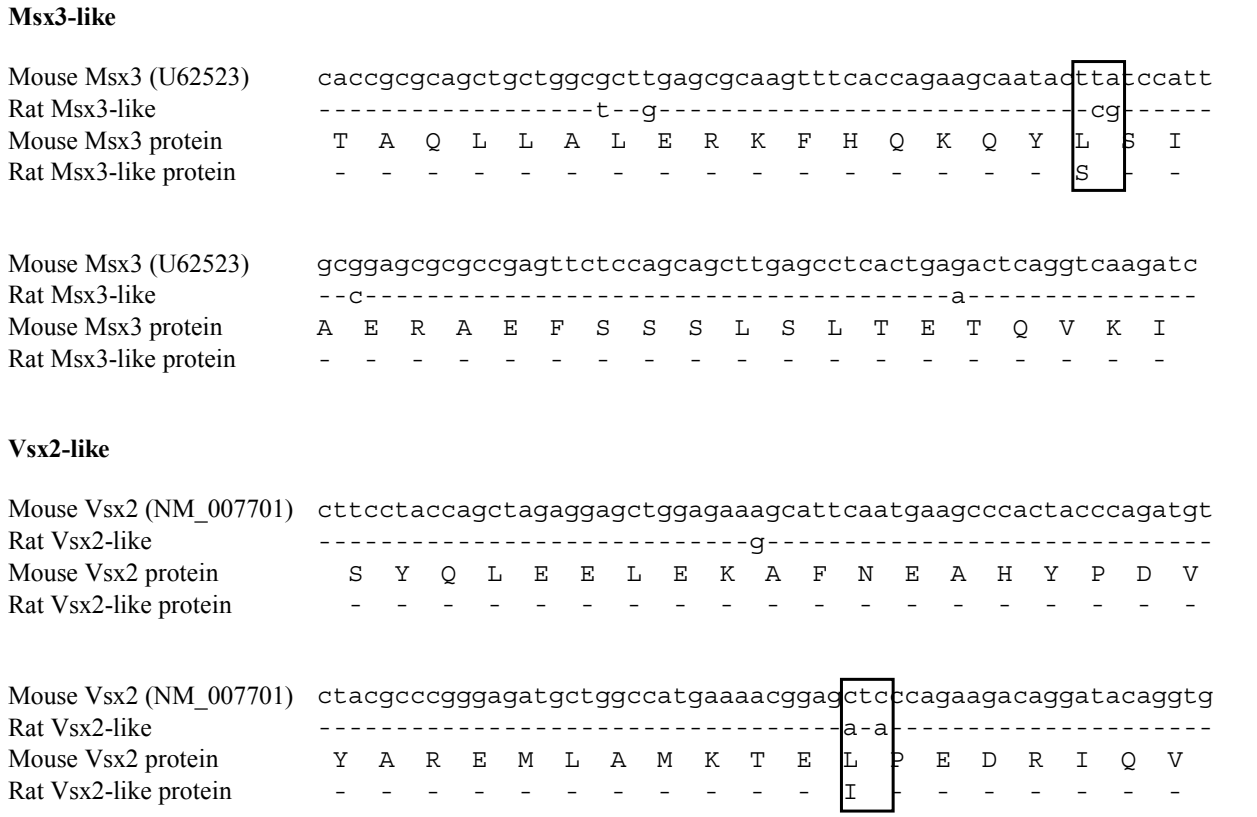

Figure 2: The two rat homeobox genes that differed from their mouse homologues
Alignment of rat *Msx3-like* and Vsx2-like to mouse *Msx3* and *Vsx2*. Hyphens indicate positions that are similar in both sequences. The rat-mouse differences in *Msx3-like* and *Vsx2-like* result in one amino acid difference each.

Cellular localization of the homeobox genes using *in situ* hybridization

In order to investigate the cellular distribution of the identified genes, we performed non-radioactive *in situ* hybridization (ISH) on frozen sections of the DRGs. Six probes showed positive staining within the DRGs (Figure 3): *Lmx1b* and *Oct1* stained diffusely in most if not all DRG neurons. *Brn3a* was also positive in all neurons, whereas *Brn3b* was only weakly expressed in a small proportion of neurons. *DRG11* was present in a subpopulation of neurons and *Prx3* was expressed in almost all neurons. Finally, *Brn2* was weakly expressed in a small group of neurons (data not shown). The other probes did not result in any staining in the DRGs, although control experiments on embryos indicated the probes to be intact. Control hybridizations using sense probes gave no signal (data not shown).

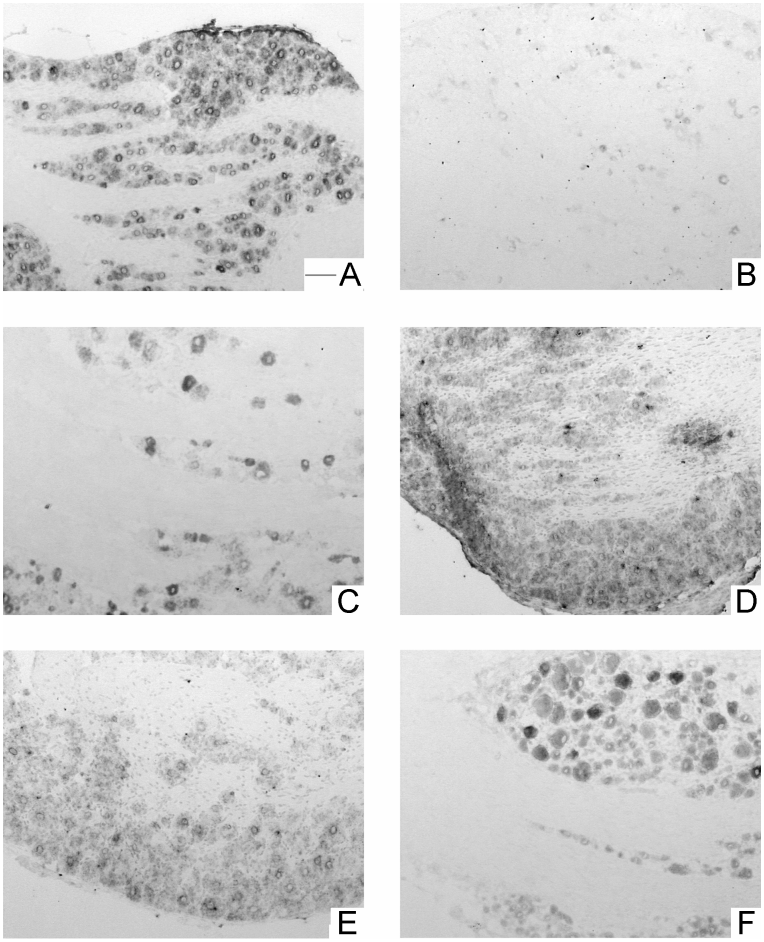

Figure 3: Expression patterns of homeobox genes in adult rat DRGs
Microphotographs of sections of the L5 dorsal root ganglion hybridized with digoxygenin-labeled antisense probes for (A) *Brn3a*, (B) *Brn3b*, (C) *DRG11*, (D) *Lmx1b*, (E) *Oct1*, and (F) *Prx3*. *Brn3a*, *Lmx1b*, and *Oct1* mRNAs are expressed in all neurons.
Brn3b is expressed very weakly in a subpopulation of neurons.
DRG11 mRNA is present in a sub-population of neurons and *Prx3* mRNA is expressed in all neurons except some large neurons.
Bar, 100 μm, applies to all panels.

DISCUSSION

In this study, a systematic characterization of the homeobox gene repertoire in adult DRGs was performed using a PCR-based strategy. We identified 21 homeobox sequences from adult rat L4-L6 DRGs. The HD PCR revealed to have a very broad scope as not only paired and paired-like homeobox genes were shown to be expressed but also members of other homeobox gene families like LIM, Msx, Hox complex, and Gbx. The POU PCR yielded only POU homeobox genes because the forward POU primer is positioned within the POU-specific domain. The PCR approach described above appeared to be a very powerful tool for a rough estimation of the homeobox gene repertoire of a certain tissue. Still, it should be expected that the actual set of expressed homeobox genes is larger, as the choice of primers introduces restricted specificity. For example, we did not find the previously described genes *Isl1* and *Oct2* [Begbie, 1996; Hol et al, 1999]. The strategy described above is also well suited for the detection of new homeobox genes. In general, homeobox genes are so well conserved between species, that the homeobox amino acid sequence is likely to be similar for rats and mice. This was the case for all our fragments except for *Msx3-like* and *Vsx2-like*. Former studies already indicated that single homeobox amino acid differences can lead to opposite functions [Smith et al, 1997]. More detailed studies should be performed, however, to further elucidate whether these genes are really different from their homologues.

We performed *in situ* hybridization in order to investigate the cellular distribution of the identified genes. In general, the staining intensity of the homeobox genes was very low. We have shown that six of the homeobox genes we detected with degenerate RT-PCR were expressed at sufficiently high levels to stain using *in situ* hybridization (ISH). *Brn3a* was positive in all neurons, whereas *Brn3b* was only weakly expressed in a small proportion of neurons. This is in accordance with Xiang *et al* (1995), who have shown Brn3a to be expressed in most of the DRG neurons and Brn3b in fewer than 50% [Xiang et al, 1995]. *DRG11* was present in a subpopulation of small to medium-sized neurons, which fits well with Saito *et al* (1995) describing *DRG11* expression in nociceptive *TrkA*-positive neurons. *Prx3* is known to be expressed in embryonic DRGs [Semina et al, 1998] and is here shown in neurons of all sizes. *Lmx1b* and *Oct1* stained diffusely in all DRG neurons and have not been reported in adult or embryonic DRG neurons before [He et al, 1989; Hobert and Westphal, 2000].

Several identified homeobox genes did not stain in the ISH experiments, but have been reported to be expressed in the neural crest or in neural crest derivatives: *Brn2*, *Gsc*, *Hoxa1*, *Msx1*, *Oct6*, *Pax3*, *Prx2*, and *Ptx2* [Conway et al, 1997; Gondré et al, 1998; Le Douarin and Kalcheim, 1999; D.B. Jacoby, personal communication]. *Brn2* stained inconsistently, indicating that the *Brn2* expression level was probably at the threshold for detection (data not shown). Although *Brn2* expression has not been described in mouse or rat DRG neurons, Liu et al (2001) have detected Brn2 protein in quail DRG neurons. *Pax3* and *Oct6* are well-known factors in Schwann cell development and have not been detected in adult Schwann cells before [Gondré et al, 1998; Zwart et al, 1996]. We found *Pax3* and *Oct6* expression in the adult DRGs, probably in Schwann cells at levels too low to detect with *in situ* hybridization. On the other hand, *Pax3* is also involved during early embryonic development in differentiation of DRG neurons [Anderson, 1999; Koblar et al 1999], so it may be that adult DRG neurons have retained low levels of *Pax3*. *Ptx2* is also expressed in embryonic DRGs at very low levels (D.B. Jacoby, personal communication). In general, it is well known that expression levels of many homeobox genes decline during maturation. Therefore, it is possible that in the adult, these genes are expressed below the detection level of *in situ* hybridization. However, it remains to be elucidated whether genes expressed at such low levels play any significant role in the adult tissue. We speculate that homeobox genes, being transcription factors, are not needed at very high levels.

For several identified homeobox genes, i.e. *Brn4*, *Gbx2*, *Gsh4*, *Hoxc5*, *Otp*, and *Zfh4* no ISH signal was obtained and their expression has not been documented before in DRGs or neural crest. The question that rises then is whether these genes were found due to contamination from spinal cord. DRGs are very well defined structures. During the process of DRG isolation, we took care to cut off the dorsal roots and to avoid touching the spinal cord. We think it would be more likely to suggest that some of the genes we found originate from connective tissue, endothelial cells or blood cells, which are also present in the DRGs, although these structures, too, were not labeled by ISH.

Finally, the two genes that could not be given definitive names, *Msx3-like* and *Vsx2-like*, could not be mapped specifically by ISH, because of the lack of appropriate probes. Mouse *Msx* genes, however, are known to be expressed in cranial neural crest derivatives [Le Douarin and Kalcheim, 1999], and we found *Msx1* as well, so it is very well possible that the Msx-family is well represented in neural crest/DRG development.

In conclusion, we identified by cloning 21 homeobox sequences from adult rat DRGs. Six of these genes positively stained DRG neurons with *in situ* hybridization. Of these, *Brn3a*, *Brn3b*, and *DRG11* are well known transcription factors in embryonic and adult DRGs. *Lmx1b* and *Oct1* have not been reported in adult or embryonic DRG neurons before. *Prx3* was shown before in developing DRGs. Seven of the identified genes have been associated before with either neural crest or embryonic DRGs and were probably too low abundant to stain. Finally, six genes were unknown in either neural crest or DRGs and two genes displayed sequence differences with their mouse counterparts, possibly being close homologues. The diversity of homeobox genes expressed in adult DRGs suggests that gene regulatory events initiated during development remain operative in the mature DRGs.

Chapter 3

Functional characterization of sciatic nerve regeneration in C57BL/6J mice

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submitted

ABSTRACT

Peripheral nerve regeneration has been studied extensively in the rat sciatic nerve crush model, both at the level of nerve function and of gene expression. In the past years, investigators started to move towards the mouse as an important experimental animal in this field, especially with respect to knock-out studies. However, there are substantial differences in the rate of regeneration between different mouse strains, and conflicting data exist on the regenerative capacity of C57BL/6J mice, a strain that is often used for the generation of knock-out mice. In this study we performed a sciatic nerve crush in C57BL/6J mice and used the expression patterns of neurotrophin receptors to discriminate between subpopulations of DRG neurons. Here we show that the mice displayed normal recovery of sensory and motor function, similar to that observed in rats. Both mice and rats developed a state of mechanical allodynia, which also recovered over time. Furthermore, we show that there is no obvious loss of specific neuronal subpopulations and that alterations in gene expression after crush are similar to those occurring in other mouse strains or in rats. These data indicate that the C57BL/6J mouse is a useful strain to monitor regeneration-related alterations in gene expression after sciatic nerve crush.

INTRODUCTION

The sciatic nerve crush model is a well-characterized model for peripheral nerve regeneration. After a crush lesion, nerve fibers in the distal stump degenerate. Myelin and axon debris are removed by the process of Wallerian degeneration. The endoneurial tubes remain intact, enabling fast and qualitatively good anatomical and functional recovery [Allt, 1976; Fawcett and Keynes, 1990; Stoll et al, 2002]. Fibers in the proximal nerve stump start to sprout and new axons extend through the distal endoneurial tube to reinnervate their target organs. During this process of axonal regeneration numerous changes in morphology (chromatolysis) and gene expression occur in the neuronal cell bodies [Aldskogius et al, 1992; Fawcett and Keynes, 1990; Hökfelt et al, 1994].

The process of regeneration has been studied extensively in the rat, both at the level of nerve function and at the level of gene expression [for review see Fu and Gordon, 1997]. In the past years, investigators started to move towards the mouse as experimental animal in this field, which provides the opportunity to relate functional aspects to expressed genes due to the availability of knock-out mice. The first regeneration-impaired knock-out mice with C57BL/6 background have already emerged [Siconolfi and Seeds, 2001; Zhong et al, 1999]. However, the C57BL/6J strain has been reported to be rather slow in nerve regeneration, as assessed by counting regenerating fibers 10-15 mm distal from the lesion [Xin et al, 1990]. Excessive cell death was proposed to underlie impaired regeneration after sciatic nerve transection in this mouse strain [Oliveira et al, 2001; Shi et al, 2001]. The transection paradigm, however, is also known to result in neuronal death in rats [Baranowski et al, 1993; Schmalbruch, 1987]. Although there are only a few reports on cell death after nerve crush, in general it is reported to be much less extensive than after transection [Baranowski et al, 1993]. Furthermore, McKay Hart et al (2002) reported that neuronal death following nerve transection was prevented by surgical nerve repair in rats, indicating that a paradigm allowing regeneration (e.g. nerve crush) would probably limit neuronal death.

Another reason for slow regeneration in C57BL/6J mice was thought to be defective recruitment of macrophages leading to the absence of Wallerian degeneration. However, the defect in Wallerian degeneration is reported in the C57BL/Ola [Brown et al, 1992] and C57BL/Wld strains

[Myers et al, 1996], which are derivatives from C57BL/6J. Groups studying these strains generally regard C57BL/6J mice as normal with respect to regeneration [Bisby and Chen, 1990; Carroll and Frohnert, 1998; Myers et al, 1996; Sommer and Schäfers, 1998]. Summarized, there are substantial differences in the rate of peripheral nerve regeneration between different mouse strains, and conflicting data exist on the regenerative capacity of C57BL/6J mice.

In this study we performed a sciatic nerve crush in C57BL/6J mice. We demonstrate that full functional recovery of sensory and motor function occurs in the C57BL/6J mouse after sciatic nerve crush similar to that observed in rats. The expression patterns of the neurotrophin receptors were used to check for differential loss of specific neuronal subpopulations in dorsal root ganglia (DRGs) and to monitor gene expression changes known to occur after sciatic nerve injury in both rats and other mouse strains. We found that there was no obvious loss of neuronal subpopulations in the DRG in C57BL/6J mice and that changes in gene expression are as expected, indicating that the C57BL/6J mouse is a useful strain to monitor regeneration-related alterations in gene expression.

MATERIALS AND METHODS

Animals and surgery

All procedures in this study were performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain [Zimmerman, 1983] and approved by the Ethical Committee on Animal Experiments of the University of Utrecht. A crush lesion was placed in both rats and mice in the sciatic nerve of the right paw, at mid-thigh level, as described by de Koning et al (1986). In short, Wistar rats (Charles River) were anesthetized with Hypnorm® (Janssen Pharmaceutics, Tilburg, the Netherlands); C57BL/6J mice (Charles River) with O_2/N_2O (1:2) and halothane. An incision was made at the thigh and the sciatic nerve was carefully exposed. At a point immediately distal from the gluteus maximus muscle, the nerve was crushed for 30 sec using a hemostatic forceps. Ten rats and six mice received a crush lesion; a similar number of animals were sham-operated. The animals were followed for 70 (rats) and 32 days (mice) and functional recovery of sciatic nerve function was monitored regularly.

For *in situ* hybridization experiments, 3 mice received a crush lesion; 3 other mice were sham-operated. After 7 post-operative days (dpo) the mice were killed by cervical dislocation, and L5 DRGs were quickly dissected and cleaned from surrounding tissue. Ipsi- and contralateral DRGs of both sham- and crush-operated animals were placed on a flat disc of frozen TissueTek, then covered with TissueTek and frozen on dry ice. Cryostat sections, cut at 8 µm thickness, were thaw-mounted onto SuperFrost®Plus slides (Menzel-Glaser), dried and stored at -80ºC. This strategy made it possible to perform stainings of ipsi- and contralateral, sham- and crush-operated DRGs on the same slide.

Functional tests

Foot reflex withdrawal test

Recovery of sensory function was measured by the foot reflex withdrawal test. A weak electric current (0.1 mA) was applied to the central portion of the foot sole using two stimulation poles (spaced 3 mm apart). Animals subjected to a nerve crush will not retract their paw upon skin contact with the poles. As reinnervation proceeds, the reflex is restored. A reaction to a 0.1 mA electric current is generally accepted as indication of complete sensory recovery [de Koning et al, 1986; van Meeteren et al, 1997]. The animals were tested daily until recovery was achieved.

Locomotor patterns

Motor function was monitored daily (mice) or 3 days per week (rats) by analysis of the walking pattern. For this purpose two strategies were used. First, hindlimb walking tracks were obtained using the de Medinacelli method by dipping both hindpaws of the animals in photographic developer (Eukobrom, Tetenal, Germany) and letting the animals walk over a strip of photographic paper (semimatt, Ilford, Paramus, NJ) [de Medinacelli et al, 1982].
Recently, a new method for gait analysis was developed by Hamers et al (2001), the CatWalk. In short, the animals traverse a walkway with a glass floor through which light is sent from the long edge. The floor functions analogous to an optic fiber in which light is completely internally reflected. Only when the paw touches the floor light is deflected and exits the glass, so that only the contact area is visible. The intensity of the spots is visualized by a DCC camera and depends on the pressure exerted, so that the spot will appear brighter when more weight is put on the paw. If no pressure is exerted on the paw, no print will be visible. Therefore, this technique is suitable to detect whether the animal supports its weight upon the paw while walking. Animals crossing the walkway are videotaped using a computer-assisted setup and digitized data are thresholded in order to extract the paw-floor contact areas and remove background (mainly a faint image of the animal due to stray light). Prints are then interactively labeled and many different parameters can be measured. Outcome parameters that are used in this study are the print area, representing the total floor area contacted by the paw during stance, and the maximum stand intensity, a measure for the mean pressure exerted during floor contact. The main difference between the de Medinacelli method and the CatWalk is that the former measures the ability to use the muscles in the lower paw and foot, and the latter shows how the paw is used during locomotion.

Mechanical withdrawal thresholds

The paw withdrawal threshold in response to a mechanical stimulus was measured two days per week in the rats using a series of von Frey filaments (Stoelting, Wood Dale IL). Pressure applied ranged from 1.14 to 18.16 g. The rats were placed in a plastic cage on a metal mesh floor and were allowed to get used to this set up prior to testing. Von Frey filaments were applied to the mid-plantar surface of the right hindpaw (when it was in contact with the floor), for 6-8 sec [Chaplan et al, 1994]. Filaments were applied in ascending order and the smallest filament eliciting a foot withdrawal response was considered the threshold stimulus.

In situ hybridization

Digoxygenin-labeled RNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Antisense and sense probes were used for the markers *TrkA* (derived from M85214), *TrkB* (derived from NM_012731), *TrkC* (derived from S60953), *cRET* (derived from X67812), and *B-50* (obtained from a cloned PCR product). The probe length varied from about 300 to 1500 bp.

In situ hybridization was carried out as described by Schaeren-Wiemers and Gerfin-Moser (1993), with minor changes. In brief, sections were dried and fixed for 10 min in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS sections were acetylated for 10 min in a solution containing 245 ml H_2O, 3.3 ml triethanolamine, 438 µl HCl (37%) and 625 µl acetic anhydride. Sections were then washed with PBS and prehybridized for 2 h at room temperature in a hybridization solution (50% deionized formamide, 5*SSC, 5*Denhardt's solution, 250 µg/ml baker's yeast RNA and 500 µg/ml herring sperm DNA). Hybridization was performed overnight at 72°C with 300-1100 ng/ml digoxygenin-labeled RNA probe in 150 µl hybridization buffer, covered with Nescofilm. The Nescofilm strips were

removed by soaking in 5*SSC at 72°C and, following washing for 2 h at 72°C in 0.2*SSC, the slides were transferred to 0.2*SSC at room temperature and washed for 5 min at room temperature with buffer 1 (100 mM Tris HCl, pH7.4; 150 mM NaCl). Preincubation with 1.5 ml buffer 1 with 10% heat inactivated fetal calf serum (hiFCS) was performed for 1 hour at room temperature in a humidified chamber. The sections were then incubated overnight at 4°C with alkaline phosphatase-conjugated mouse anti-digoxygenin Fab fragment (Boehringer Mannheim), 1:5000 diluted in buffer 1 with 1% hiFCS. Following washing with buffer 1 and equilibration with buffer 2 (100 mM Tris HCl, pH9.5; 50 mM $MgCl_2$; 100 mM NaCl), the color reaction was performed in the dark for 24 h at room temperature with 200 µl NBT/BCIP solution (Boehringer Mannheim) and 2.4 mg levamisole in buffer 2. The slides were then washed with TE buffer and H_2O, dehydrated and coverslipped with Entellan.

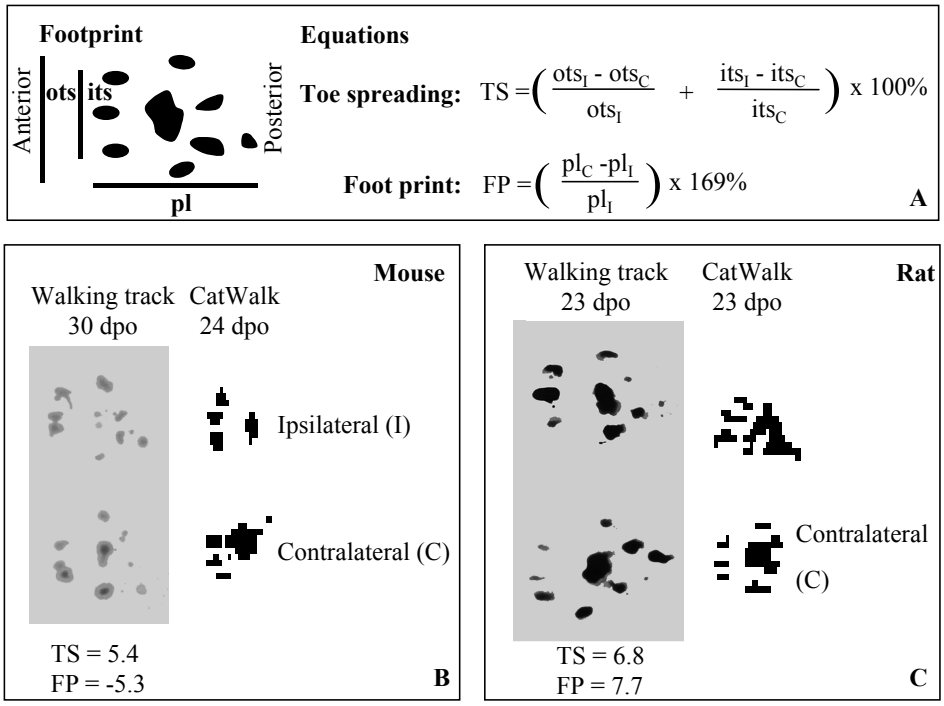

Figure 1: Walking track analysis in mice and rats
Panel A shows a cartoon of the footprint with the parameters that are important for analysis and the equations used for calculating the toe spreading and footprint from the ipsilateral paw (I) relative to the contralateral (C) paw. Toe spreading (TS) is a measure for the function of the intrinsic muscles of the paw. The print length (pl) reflects the functions of the soleus muscle and the gastrocnemic muscles, which are among others involved in raising of the ankle [de Koning and Gispen, 1987]. The factors 100 and 169 set the TS and FP at –100% immediately after crush. The TS and FP are regarded as normal when they are within the range of –10 to 10% [de Koning and Gispen, 1987]. its = inner toe spreading; ots = outer toe spreading; pl = print length.
Panel B shows the walking tracks of the mice, assessed with the photographic papers (left) at 30 dpo, and the subthreshold CatWalk prints at 24 dpo (right, outer toes not visible). Toe spreading and footprint were normal, with no placement of the ankle on the floor. Panel C shows the walking tracks and subthreshold prints for rats at 23 dpo for rats. Similar to the mice, the walking tracks of the rats showed recovery of toe spreading and footprint.

RESULTS

Recovery of sensory and motor function

Recovery of sensory function was assessed using the foot reflex withdrawal test. At 18 dpo, 5 of the 6 mice and at 20 dpo all mice reacted to the 0.1 mA electric current. The mice tended to generate very poor walking tracks with the de Medinacelli method, therefore, we only made walking tracks from the mice at 30 dpo. At that time point, the prints of the lesioned paws were normal with respect to toe spreading and print length (Figure 1B).
The automated CatWalk paradigm showed that the maximum stand intensity significantly decreased after crush, and was around 75% at 25 dpo (Figure 2B). The stand intensity gradually recovered and was not significantly different from the sham-operated paw from onward 28 dpo. The print area was 30% at 25 dpo and then gradually recovered but remained different from the sham-operated mice throughout the 32 days (Figure 2A). From the digital CatWalk recordings, we were able to extract sub-threshold prints at 24 dpo, which showed no obvious difference in toe spreading and footprint (Figure 1B), indicating functional reinnervation of the intrinsic foot muscles.

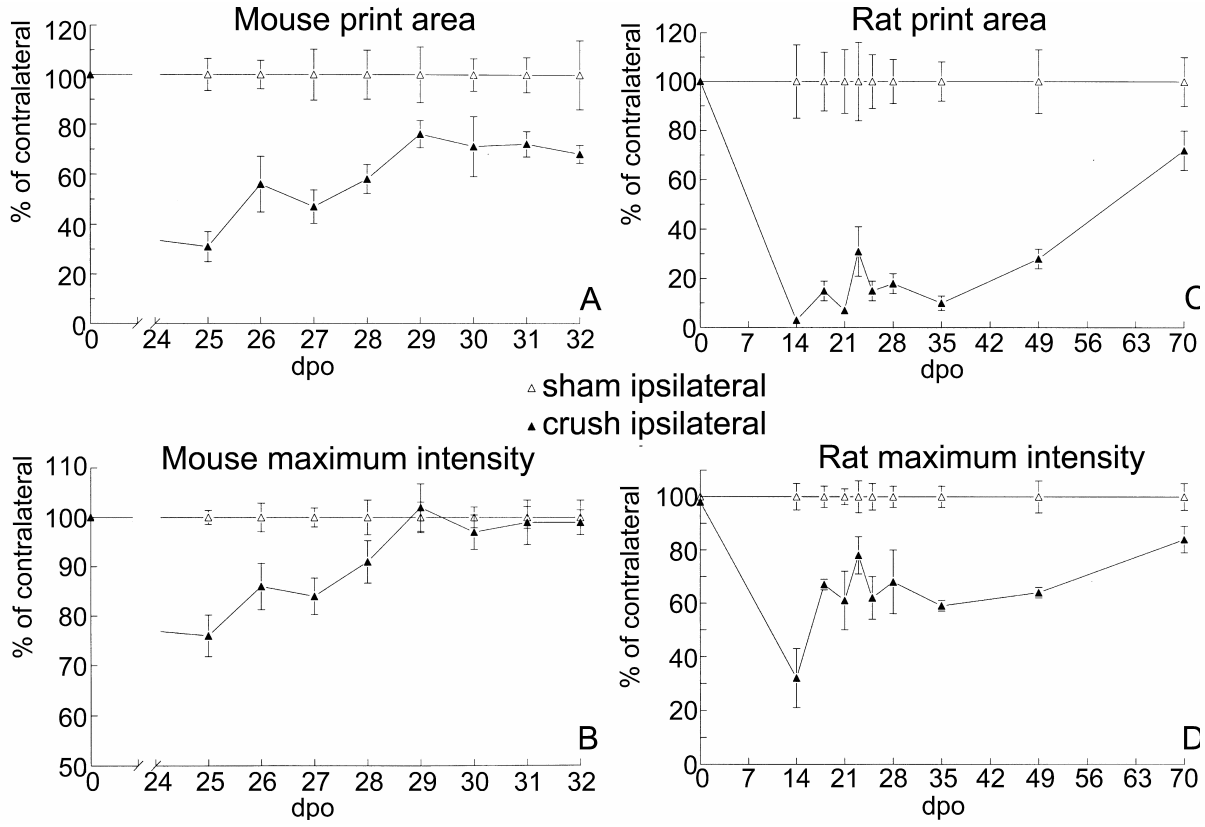

Figure 2: CatWalk analysis in mice and rats
Print area and maximum stand intensity of ipsilateral sham- and crush-operated paws shown as a percentage of the contralateral paws during the follow-up period of 32 days in mice (A,B) and 70 days in rats (C,D).

At 20 dpo 7 of the 10 rats and at 21 dpo all rats reacted to the 0.1 mA electric current, indicating recovery of sensory function. The de Medinacelli method showed that motor function was recovered in the rats at 23 dpo: the footprint en toespreading parameters were normal (Figure 1). However, the automated CatWalk paradigm revealed that at this time point the rats did not put

much pressure on the ipsilateral paw (Figure 2D). After the initial decrease, the maximum stand intensity and the print area increased progressively, but were still not normal at 70 dpo (Figure 2C and D).

These observations point to a state of neuropathic pain [Vrinten et al, *in press*], which we investigated in the rats measuring the response to mechanical stimuli using von Frey filaments. Indeed, recovery of the CatWalk parameters paralleled the response of the rats to von Frey filaments. The withdrawal threshold of the operated hindpaw decreased, indicating the presence of mechanical allodynia. The first significant threshold decrease was observed at 21 dpo, which correlates with the time point of recovery of sensory function. The threshold remained low for 2 weeks and generally increased. At 70 dpo the threshold was still slightly (but significantly) different as compared to the sham-operated animals (Figure 3), an observation that correlated with the CatWalk data.

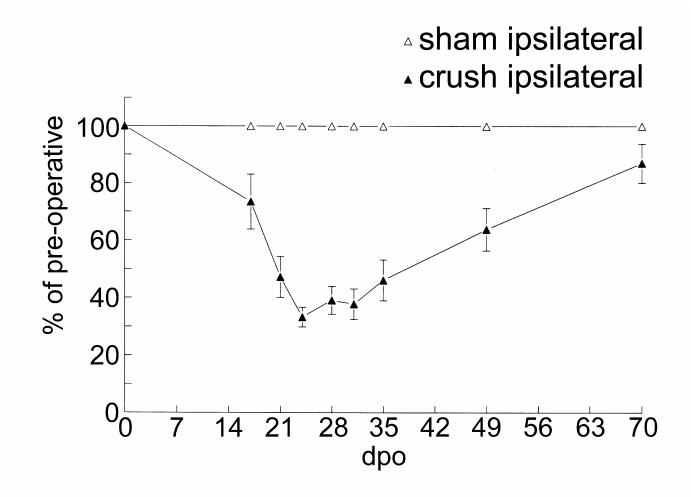

Figure 3: Mechanical allodynia in cruh-lesioned rats
Mechanical withdrawal thresholds as determined by application of von Frey filaments in sham- and crush-operated rats during the follow-up period of 70 days. Data are presented as percentages of the pre-operative values.

In situ hybridization using markers for specific neuronal subpopulations

Figure 4 shows the results of the *in situ* hybridization with *TrkA*, *TrkB*, *TrkC*, and *cRET* on DRGs of C57BL/6J mice at 7 dpo. Ipsi- and contralateral DRGs of the sham-operated mice and contralateral DRGs of the crush-operated mice were used as controls on the same slide as the ipsilateral crush-operated DRGs. *TrkA* was expressed at high levels mainly in small to medium-sized and in some large neurons. The distribution of *TrkA*-positive neurons in the ipsilateral crush-lesioned DRGs was similar, but the expression levels were lower as compared to the contralateral and sham-operated DRGs. *TrkB* mainly labeled satellite cells, surrounding the neurons. Some medium-sized neurons also expressed *TrkB*. No difference was observed between crush- and sham-lesioned DRGs. *TrkC* was expressed in medium to large neurons and was also not affected by the crush lesion. Finally, *cRET* stained neurons of all sizes in the sham- and in the contralateral crush-operated DRGs. In the ipsilateral crush-operated DRGs more large-sized *cRET*-positive neurons were observed.

We used *B-50* as a positive control for the regeneration-associated changes in gene expression. Expression of *B-50* was characteristically low in the contralateral and sham-operated DRGs, being confined to small- to medium-sized neurons. *B-50* was rapidly upregulated after crush, both in small to medium neurons, where expression increased, and in large neurons, where expression of *B-50* was reinduced.

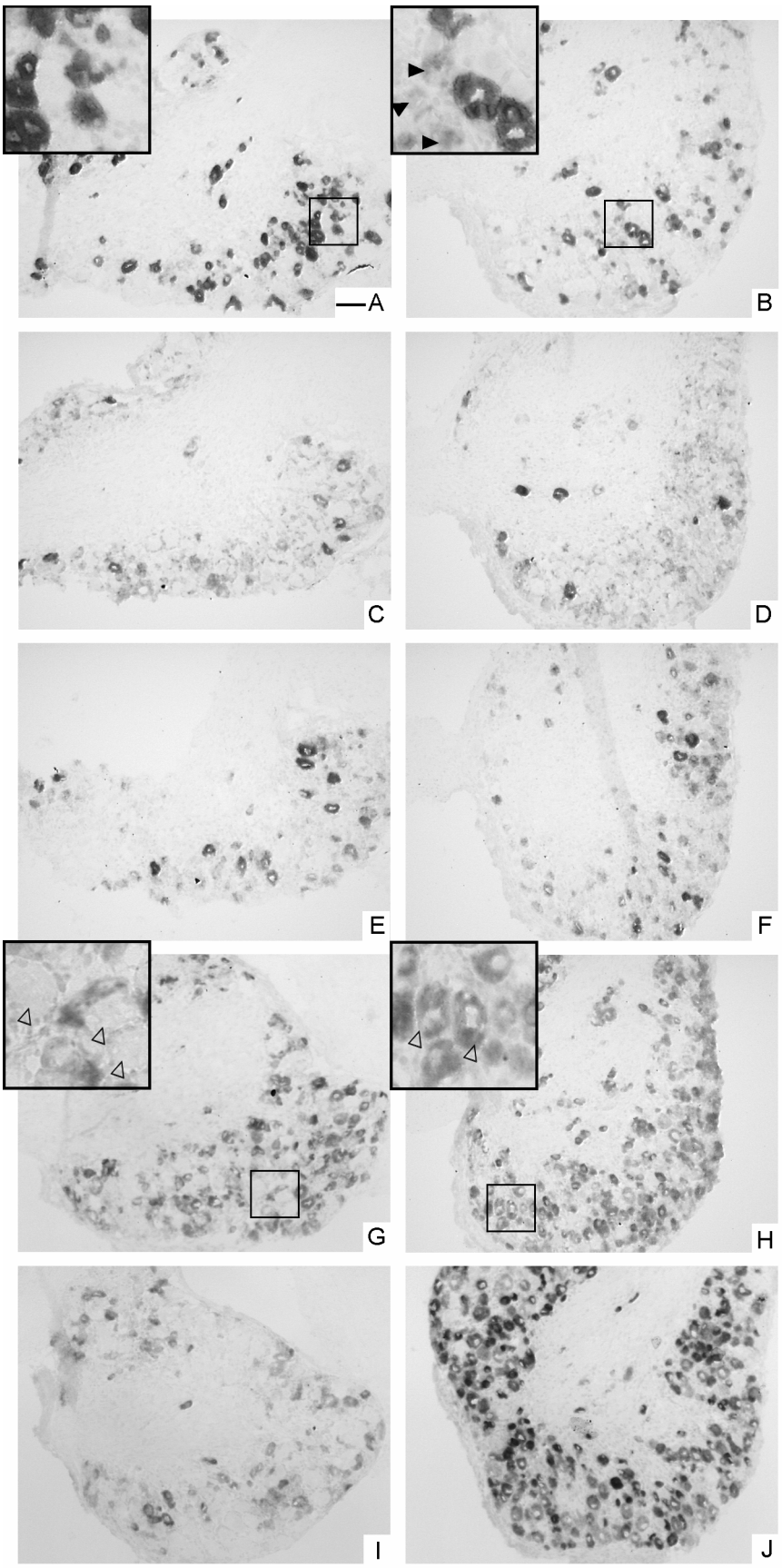

Figure 4:
Expression patterns of DRG neuronal markers
In situ hybridization on crush-lesioned contralateral (left column) and ipsilateral (right column) DRGs at 7 dpo using probes for *TrkA* (A,B), *TrkB* C,D), *TrkC* (E,F), *cRET* (G,H), and *B-50* (I,J).
Insets: larger magnifications of areas of interest, showing that *TrkA* (A,B) is expressed in a similar number of cells but at lower levels (filled arrowheads) and that *cRET* (G,H) is expressed in more large-sized neurons (open arrowheads) in ipsilateral DRGs.

DISCUSSION

Because of the conflicting data that currently exist on the regenerative capacity of C57BL/6J mouse strain, and the frequent use of this strain for studies on regeneration in knock-out mice, we studied the effects of sciatic nerve crush on DRG neurons in C57BL/6J mice. We compared recovery of sensory and motor functions in C57BL/6J mice to that in rats. The footflick withdrawal test showed that sensory function in the mice had returned to normal at 20 dpo, slightly earlier than in rats, which had all recovered by 21 days. The walking tracks, obtained using photographic paper, showed that the motor function of the rats was normal at 23 dpo, slightly after the return of sensory function. Although we only had walking tracks of the mice at 30 dpo, we assumed that motor function of the mice also recovered around this time point of return of sensory function. The subthreshold signals of the CatWalk measurements indeed showed normal footprints at 24 dpo. Other studies on mice also showed recovery of motor function around 24 dpo [Verdú and Navarro, 1997; Zhong et al, 1999].

Although these data indicate that recovery of motor function coincided with recovery of sensory function, the CatWalk system showed that the animals did not support their weight on the lesioned paw. The print area, although recovered on the photographic films, where one does not need much pressure to get a print, did not return to normal in either rats or mice during the follow-up period using the CatWalk system. This reduction in pressure applied during stance was also shown by Vrinten and Hamers (*in press*) in rats with chronic constriction injury (CCI), a model for neuropathic pain. Indeed, the response of crush-lesioned rats to von Frey filaments indicated that following recovery of sensory (and motor) function, the rats developed mechanical allodynia, a type of neuropathic pain in which normally non-painful stimuli lead to a pain response. The von Frey measurements and the CatWalk data corresponded, both showing that at 70 dpo there was still a hypersensitivity of the previously injured paw. This is consistent with Vrinten and Hamers (*in press*) who showed that pressure estimates recorded by these methods correlated quite well in the CCI-model for chronic pain.

Neuropathic pain after peripheral nerve transection as well as chronic constriction injury (CCI) is thought to be caused by sympathetic sprouting primarily around large neurons [Hu and McLachlan, 2001; Jones et al, 1999; Shinder et al, 1999; Zhou et al, 1999] and by aberrant sprouting of A-fibers into lamina II of the spinal cord [Mannion et al, 1996]. These structural changes coincide with an upregulation of the neuropeptides SP and CGRP in large mechanosensory neurons as opposed to small nociceptive neurons, which downregulate SP and CGRP [Hökfelt et al, 1994; Hu and McLachlan, 2001; Noguchi et al, 1994; Woolf et al, 1992]. These changes are thought to lead to pain signaling of larger mechanosensory neurons in response to normally non-painful mechanical stimuli [Campbell, 2001]. Furthermore, proinflammatory cytokines produced by Schwann cells and invading macrophages are thought to play a role in pain induced by nerve injury [Watkins and Maier, 2002]. It is likely that the mechanical allodynia observed in the crush-lesioned rats follows the same mechanisms as in the transection/CCI models. The CatWalk data in the mice indicate that, similar to rats, the mice also developed mechanical allodynia after nerve crush, but they recovered faster than the rats. The maximum intensity did reach control levels in the mice, after 28 days. This may be due to the smaller weight and the faster gait of mice.

To check whether all neuronal populations were still present, we performed *in situ* hybridization with probes for *TrkA*, *-B*, *-C*, and *cRET*, the receptors for NGF, BDNF, NT-3, and GDNF, respectively. During development, specific subpopulations of sensory neurons depend on these

neurotrophic factors and their receptors mark roughly all neuronal subtypes in the DRGs [Bibel and Barde, 2000; Molliver, 1997]. We observed roughly equal cell numbers in sections through the DRGs from unilaterally crush- or sham-operated mice at 7 dpo. The number of neurons expressing *TrkB* and *TrkC* did not change, whereas the intensity, but not the number, of *TrkA*-positive neurons was lower in ipsilateral crush-lesioned DRGs than in contralateral crush-lesioned and sham-operated DRGs. In ipsilateral crush-lesioned DRGs an increase in *cRET*-positive neurons was observed. These findings were consistent with other studies on neurotrophic factor receptor expression in rats or other mouse strains [Bennett et al, 2000; Mohiuddin et al, 1999; Naveilhan et al, 1997; Verge et al, 1996]. Our data on *TrkB* expression are consistent with Kashiba et al (1996) showing a relatively small population of neurons (3-8%) positive for *TrkB* mRNA. Foster et al (1994) reported using immunohistochemistry a much larger number of TrkB-immunoreactive neurons and a rise after injury. Because of the large number of TrkB-immunoreactive neurons they observed in the uninjured state, we cannot compare our data with their study. To date, no mRNA data on *TrkB* expression in DRG neurons after sciatic nerve crush have been published. As a positive control for the regeneration-associated changes in gene expression, we performed *in situ* hybridization with *B-50* (also called *GAP-43*). The upregulation of *B-50* expression that is characteristic for nerve regeneration in the rat [Oestreicher et al, 1997; Plantinga et al, 1993a; van der Zee et al, 1989; Woolf et al, 1990] was reproduced in the mouse DRGs in our experiment. We conclude that in the C57BL/6J mouse strain, there is no obvious loss of specific neuronal subpopulations and alterations in gene expression during regeneration occur similar to those occurring in other mouse strains or in rats.

In conclusion, we showed that the mice displayed normal recovery of sensory and motor function, similar to recovery in rats. Both mice and rats developed a state of mechanical allodynia, which also recovered as regeneration proceeded. Furthermore, we showed that there is no obvious loss of specific neuronal subpopulations after sciatic nerve crush in the C57BL/6J mouse strain and that alterations in gene expression during regeneration occur similar to those occurring in other mouse strains or in rats. These data indicate that the C57BL/6J mouse is a useful strain to monitor regeneration-related alterations in gene expression after sciatic nerve crush.

Chapter 4

Developmental patterns of homeobox gene expression in DRG neurons are not recapitulated during regeneration of the crushed sciatic nerve

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submitted

ABSTRACT

The adult peripheral nervous system is able to regenerate after injury. Regeneration is associated with the expression of new genes and proteins. Proteins abundant in developing axons increase in expression after injury, whereas proteins involved in neurotransmission are downregulated. It has been hypothesized that molecular mechanisms underlying regeneration-associated alterations in gene expression may be a recapitulation of developmental processes. These gene expression changes are likely to be regulated by changes in the gene expression of transcription factors. As homeobox genes play important roles in embryonic development of the nervous system, it makes them candidates for a regulatory role in the process of regeneration. In a previous study, we characterized the homeobox gene repertoire of the adult rat dorsal root ganglia (DRGs), which contain sensory neurons. In the present study, we first screened for homeobox genes that might display altered expression in the well-established rat sciatic nerve crush model. Next, we quantified selected genes *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp* and *Pax3*, during regeneration of the sciatic nerve in the mouse using real-time quantitative PCR. Here we show that the relative mRNA expression levels of *Isl1* decreased shortly after crush, but those of *DRG11*, *Lmx1b*, and *Pax3* did not change after crush. *Gsc* and *Otp* were highly variable between and within experimental groups. These data indicate that the developmental expression patterns of the homeobox genes studied here are not recapitulated during regeneration of the DRG neurons. We conclude that developmental gene expression programs controlled by these homeobox genes are not directly involved in sciatic nerve regeneration.

INTRODUCTION

Neurons of the adult peripheral nervous system are able to regenerate their peripheral axons after injury. The sciatic nerve, which innervates the hindpaw of the animal, is frequently used as a model to study peripheral nerve regeneration. After crush injury, the fibers distal to the lesion undergo Wallerian degeneration: the axons and myelin sheets degenerate, and are ingested by Schwann cells and invading macrophages. Schwann cells surrounding the distal fibers proliferate so that the endoneurial tubes surrounding the original nerve fibers remain intact, providing the environment through which the regenerating axons can grow. Sensory neurons in the dorsal root ganglia (DRGs) make new fibers that regrow into the endoneurial tubes, a regenerative process that leads to complete recovery [Bridge et al, 1994; Fawcett and Keynes, 1990; Stoll et al, 2002].

Regeneration is associated with the expression of new genes and proteins. Several studies indicated that, in mature sensory neurons undergoing regeneration, the developmental phenotype is reinduced. Proteins abundant in developing axons increase in expression after crush, whereas proteins involved in neurotransmission are downregulated [Aldskogius et al, 1992; Fawcett and Keynes, 1990; Hökfelt et al, 1994]. Cytoskeletal proteins, like tubulin and actin, are massively produced and transported to the injured axon [Aldskogius, 1992; Fawcett and Keynes, 1990]. B-50 (also called GAP-43), a growth-associated protein that is present in embryonic growth cones, is also upregulated as well as proteins that are involved in axonal pathfinding during development [Plantinga et al, 1993a; van der Zee et al, 1989; Woolf, 1990; Zhang, 2000].

Several neuropeptide genes, like substance P (SP) and calcitonin gene-related peptide (CGRP), are decreased after nerve transection, which is generally regarded as a downregulation of the functional (transmitting) phenotype of the neurons [Bergman et al, 1999; Hökfelt et al, 1994].

These alterations in DRG neuronal gene expression after sciatic nerve injury were hypothesized to be a recapitulation of developmental processes [Fawcett and Keynes, 1990; Skene, 1989; Wong and Oblinger, 1990].

The molecular mechanisms underlying regeneration-associated alterations in gene expression are likely to be driven by transcription factors. Homeobox genes encode transcription factors that play important roles in development of the (peripheral) nervous system [Akopian et al, 1996; Anderson, 1999; De Robertis, 1994; Gehring, 1998]. Since it has been hypothesized that regeneration may be based on recapitulation of developmental mechanisms, we focused on homeobox gene expression in the DRGs during sciatic nerve regeneration.

Some reports already indicated changes in homeobox gene expression during regeneration. Particularly, Schwann cells in the distal stump dedifferentiate and reinitiate their developmental program, re-expressing the POU class homeobox gene *Oct6/SCIP* and the paired class gene *Pax3* [Küry et al, 2001a]. Members of the *Hox* complex, *Hoxb5*, *Hoxd3*, and *Hoxa6*, were reported to decline in expression in Schwann cells after nerve crush [Gondré et al, 1998; Küry et al, 2001a; Sherer et al, 1994]. In DRG neurons, it has been described that following sciatic nerve injury, the LIM homeobox gene *Isl-1* is slightly decreased at 7 days after injury [Hol et al, 1999] and the POU homeobox gene *Oct2* is increased [Begbie et al, 1996].

In a previous study, we identified 22 homeobox genes expressed in adult DRGs of the rat [Vogelaar et al, *in press*]. In the present study, we used a semi-quantitative approach to screen for those homeobox genes that display altered expression after sciatic nerve crush. Next, we determined the regulation of selected genes, *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp*, and *Pax3* in more detail in the mouse using real-time quantitative PCR. Here we show that the developmental expression patterns of the homeobox genes studied are not recapitulated during regeneration of the DRG neurons. We conclude that the regeneration process does not depend on a recapitulation of the developmental homeobox gene expression repertoire but involves separate mechanisms.

MATERIALS AND METHODS

Experimental animals and surgery

All animal procedures were performed in accordance with the Ethical Committee on Animal Experiments of the University of Utrecht. We used the rat sciatic nerve crush model to screen for homeobox genes that might display altered expression during regeneration. Six-weeks-old male Wistar rats (Wistar: Unilever, Central Animal Faculty, Utrecht University, the Netherlands), weighing 120-140 g were housed under standard conditions. The rats were anaesthetized with Hypnorm (Philips Duphar, Amsterdam, the Netherlands) and had a unilateral mid-thigh sciatic nerve crush or a sham operation [de Koning et al, 1986]. Eighteen rats received a unilateral crush lesion of the sciatic nerve at mid thigh level and were killed by decapitation after 1 (n=6), 4 (n=6), and 7 (n=6) post-operative days (dpo) survival time. Six rats were sham-operated and killed by decapitation after 1 day. Ipsilateral DRG L4-L6 were quickly dissected, cleaned from surrounding tissue, pooled per group and frozen on dry ice for RNA isolation.

For real-time quantitative PCR, six-weeks-old male C57BL/6J mice (Charles River) were housed under standard conditions. The mice were anaesthetized with O2/N2O (1:2) and halothane and received a unilateral mid-thigh sciatic nerve crush or a sham operation. Twenty-seven mice had a crush lesion and were killed by cervical dislocation after 1 (n=9), 4 (n=9), and 7 (n=9) post-

operative days (dpo) survival time. Twenty-seven control mice were sham-operated and were killed at similar time points. Contra- and ipsilateral DRG L4-L5 were quickly dissected, cleaned from surrounding tissue, and frozen on dry ice for RNA isolation. In order to get sufficient amounts of material the DRGs of 3 mice were pooled, resulting per experimental group in 3 vials with ipsilateral DRGs and 3 vials with contralateral DRGs.

Screening for regeneration-associated homeobox gene expression

Total RNA was extracted from the rat DRGs following the TRIzol isolation method (Life Technologies) according to the manufacturer's protocol, treated with DNase and subjected to RT-PCR with degenerate primers, as described previously [Vogelaar et al, *in press*]. The PCR products were loaded on a 1% agarose gel and electrophoresed at 80V for 45 min. Blots were made applying the downward blot procedure. Probes were synthesized by labeling specific homeobox sequences with ^{32}P using the Random Primed DNA Labeling Kit (Boehringer Mannheim). The blots were prehybridized at 65°C for 1 hour with hybridization solution (0.5 M $NaHPO_4$, pH 7.2; 1% BSA; 1 mM Na_2EDTA; 7% SDS). Then, the probes were denatured by heating and added to the hybridization solution. Hybridization was performed overnight at 65°C. The blots were washed at 65°C for 15 min with hybriwash5 (40 mM $NaHPO_4$, pH7.2; 1 mM Na_2EDTA; 5% SDS) and 4 times at 65°C for 20 min with hybriwash1 (40 mM $NaHPO_4$, pH7.2; 1 mM Na_2EDTA; 1% SDS). Biomax MR films were exposed for 1-3 h, in some cases for 16-60 h.

Quantification of homeobox genes using real-time quantitative PCR

Total RNA was extracted from the mouse DRGs following the TRIzol isolation method (Life Technologies) according to the manufacturer's protocol. To ensure purity of the RNA an additional chloroform extraction step was performed and after the procedure the RNA was purified with sodium chloride precipitation. First strand cDNA synthesis was performed on 2 µg of total RNA in a final volume of 100 µl containing 1x first strand buffer, 0.01 M DTT, 750 ng random hexamers, 250 U Superscript II RNase H- reverse transcriptase, 500 µM dNTPs and 36 U RNAguard and incubated at 42°C for 50 min. Reverse transcriptase was inactivated for 10 min at 70°C and the volume was increased to 200 µl.

Primers and fluorogenic probes for *cyclophilin*, *B-50*, *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp*, and *Pax3* (Table 2) were designed using Primer Express software (PE Biosystems), according to the manufacturer's guidelines as described previously [Bogerd et al, 2001]. Regarding the homeobox genes the positions of primers and probes were chosen such that the homeobox was not included in the PCR. Each set of primers was first tested on mouse E14.5 cDNA and products were sequenced to check the specificity. Products were cloned into pGEM-T easy vectors following the manufacturer's protocol and colony PCRs were performed with primers directed to the T7 and SP6 sites in the vector. For optimization 10^4 copies of the purified colony PCR products were produced and used as templates to determine the optimal concentration of primers and probes (Table 2). Experiments to check the efficiencies of the PCRs were performed on cDNA dilutions of 10^0 to 10^{-3} for *cyclophilin*, *B-50*, *DRG11*, *Isl1*, and *Lmx1b* or on a series of 10^4 to 10^1 copies for *Gsc*, *Otp*, and *Pax3*.

To assess the relative expression levels of the homeobox genes, Taqman PCR assays were performed in triplicate on 5 µl of cDNA in a 25 µl reaction volume containing 1x Taqman Universal PCR Master Mix (Applied Biosystems) and the appropriate concentration of primers and probes. The ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used and

data were collected using the Sequence Detection Software (version 1.6.3) provided by the manufacturer. For every sample, an amplification plot was generated, showing the increase in the reporter dye fluorescence with each PCR cycle. The reporter signal was normalized to the fluorescence of an internal reference dye. From each amplification plot, a threshold cycle (C_t) value was determined, representing the PCR cycle number in the exponential phase of the PCR [Bustin, 2000]. The C_t value is inversely proportional to the log of the initial mRNA copy number.

To correct for differences in cDNA load between the samples, the target PCRs (i.e. the homeobox gene PCRs) were normalized to the reference PCR, *cyclophilin*, a housekeeping gene [Medhurst et al, 2000]. The expression levels of the target genes ($X_{0,target}$) relative to *cyclophilin* ($X_{0,ref}$) were calculated using the equation $X_{0,target}/X_{0,ref} = E^{-Ct,target}/E^{-Ct,ref}$ in which E is the PCR efficiency of the individual genes (Table 2) as described by Kamphuis et al (2001). We used the $X_{0,target}/X_{0,ref}$ values of the sham-operated contralateral DRGs per time-point as a calibrator by dividing the $X_{0,target}/X_{0,ref}$ values of each of the samples with the mean $X_{0,target}/X_{0,ref}$ value of the three contralateral sham samples. The resulting value represents the target gene expression levels of the ipsilateral sham-operated and the ipsi- and contralateral crush-operated DRGs relative to the contralateral sham-operated DRGs.

In situ hybridization

For *in situ* hybridization, 3 mice received a crush lesion, 3 other mice were sham-operated. At 1, 4, and 7 dpo, ipsi- and contralateral DRGs corresponding to spinal level L5 were quickly dissected and cleaned from surrounding tissue. Per time point ipsi- and contralateral DRGs of both sham- and crush-operated animals were placed on a flat disc of frozen TissueTek, then covered with TissueTek and frozen on dry ice. Cryostat sections, cut at 8 µm thickness, were thaw-mounted onto SuperFrost®Plus slides (Menzel-Glaser), dried and stored at –80ºC. This strategy made it possible to perform *in situ* hybridization on ipsi- and contralateral, sham- and crush-operated DRGs on the same slide. Digoxygenin-labeled RNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Antisense and sense probes were used for *B-50* obtained from a cloned PCR product. The probe length was 265 bp. In situ hybridization was carried out as described before [Vogelaar et al, *in press*].

RESULTS

Screening for regeneration-associated homeobox genes

We investigated in rat DRGs after sciatic nerve crush whether regeneration-associated alterations occurred in the expression of homeobox genes, identified before in non-lesioned DRGs [Vogelaar et al, *in press*]. Using a semi-quantitative approach, we observed that the majority of the homeobox genes expressed in adult DRGs did not change after crush. The data suggested that only *Otp*, *Gsc* and *Vsx2-like* were increased, whereas *Lmx1b* was decreased (Table 1). To follow up this screening, we aimed to quantify the expression of these genes during the regeneration process using real-time quantitative PCR. Because of the availability of mouse gene sequences in the databases and the potential to use mouse mutants as experimental animal in regeneration experiments, we switched to the mouse sciatic nerve crush model.

Table 1: Homeobox gene expression in regenerating rat DRGs

Homeobox gene	Semi-q
DRG11	-
Gbx2	-
Gsc	↑
Gsh4	-
Hoxa1	-
Hoxc5	-
Lmx1b	↓
Msx1	-
Msx3-like	-
Otp	↑
Pax3	-

Homeobox gene	Semi-q
Prx2	-
Prx3	-
Ptx2	-
Vsx2-like	↑
Zfh4	-
Brn2	-
Brn3a	-
Brn3b	-
Brn4/RHS2	-
Oct1	-
Oct6	-

The homeobox genes identified using semi-quantitative measurements in sham- and crush-operated rat DRGs.
- = no change, ↑ = upregulated, ↓ = downregulated

Validation of the quantitative PCR

We performed quantitative PCR using specific primers and Taqman probes (Table 2). We initiated the studies using three housekeeping genes, *cyclophilin*, *glyeraaldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*), in order to choose the housekeeping gene that fitted best for these experiments. Expression of *GAPDH* revealed to be variable, which is not surprising because of the changed metabolic state of the neurons [Fawcett and Keynes, 1990; Lieberman, 1971]. For *HPRT*, we obtained results similar to *cyclophilin*, indicating that both genes are suitable as reference genes in the regeneration paradigm.

Here we show the quantification of the growth-associated protein *B-50* and of the homeobox genes - *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp*, and *Pax3* - relative to the levels of the housekeeping gene *cyclophilin*. We first determined the efficiencies of the individual PCRs. Ten-fold dilutions (10^0 - 10^{-4}) of mouse DRG cDNA were used for this purpose. The measured cycle threshold (C_t) plotted against the logarithm of the cDNA concentration was used to calculate the PCR efficiencies (E) using the equation $E = 10^{-(1/slope)}$ [Kamphuis et al, 2001]. Figure 1 shows the plots for *cyclophilin*, *B-50*, *DRG11*, *Isl1*, and *Lmx1b* with slopes ranging from -3.4 to -3.56, resulting in efficiencies of 1.91 to 1.97 (Table 2). Expression levels of *Gsc*, *Otp*, and *Pax3*, however, were extremely low in undiluted cDNA – C_t's ranging from 35 to 38 (Figure 1) - making it impossible to determine efficiencies of these PCRs on cDNA dilutions. Therefore, the efficiency measurements of these PCRs were performed on dilutions of 10^4 to 10^1 copies of PCR product produced by colony PCR on the cloned PCR fragments. The efficiencies of the *Gsc*, *Otp*, and *Pax3* PCRs (Figure 1) were between 1.93 and 2.02 (Table 1, Figure1). To check whether the efficiencies were constant with different cDNA loads, we calculated the $X_{0,target}/X_{0,ref}$ in the dilution series of cDNA or copies. The $X_{0,target}/X_{0,ref}$ were not significantly different between the dilutions, indicating that the efficiencies of the reference and the target genes were constant (data not shown).

Table 2: Sequence, positions, and properties of primers and probes used for quantitative PCR

Gene	Accession number	Primer	positions	Sequence 5'- 3'	Optimal conc. (nM)	PCR Efficiency	$X_{0,target}/X_{0,ref}$ naive DRG
cyclophilin	NM_008907.1	forward	19-35	GCTTTTCGCCGCTTGCT	300	1.91	
		reverse	86-69	CTCGTCATCGGCCGTGAT	300		
		probe	43-66	TGGTCAACCCCACCGTGTTCTTCG	200		
B50	NM_008083	forward	234-253	GCTCATAAGGCTGCGACCAA	900	1.97	2.1E-02
		reverse	306-286	TCTCGCCTTTGAGCTTTTTCC	900		
		probe	256-284	TTCAGGCTAGCTTCCGTGGACACATAACA	300		
DRG11	AY116506	forward	74-95	GCAGAAATCGAACGACCTTCAC	900	1.97	3.6E-02
		reverse	158-133	GTGAAGACATCTGGGTAGTGTGTTTG	900		
		probe	123-100	TGCCTCCAGAGCTTCCAGCTGCTG	100		
Gsc	NM_010351.1	forward	939-962	TTGCACAGACAGTCGATGCTACTT	900	1.93	1.1E-06
		reverse	1037-1014	TCCTGGGCCTGTACATTATTTACA	900		
		probe	984-964	CACACACCCTGCCTTGCCGGGA	200		
Isl1	NM_021459.1	forward	881-905	AACCCAACGACAAAACTAATATCCA	900	1.95	3.0E-02
		reverse	986-946	GCCTGTAAACCACCATCATGTCT	900		
		probe	934-911	CAGCCACCATGGGAGTTCCTGTCA	200		
Lmx1b	NM_010725.1	forward	119-137	TCTCCGACCGCTTCCTGAT	300	1.92	1.7E-03
		reverse	205-187	TGGTGAGGGCTTGCTGACA	300		
		probe	179-155	CACTGCAAACACTCCTCGTGCCAGG	200		
Otp	NM_011021.1	forward	87-112	AATAACTTAAAGCCGCTAAAATTTGG	900	1.96	2.1E-06
		reverse	167-150	GGAGGTCGGCGTGAGACA	900		
		probe	143-126	CGCACCGCTCCAGGGCGA	200		
Pax3	NM_008781.1	forward	841-864	AAAAAGGCTAAACACAGCATCGAT	300	2.02	1.1E-06
		reverse	914-894	TCGGAGCCTTCAACTGACTGA	300		
		probe	869-892	TCCTGAGTGAGCGAGCCTCTGCAC	200		

Sequences are shown for the forward and reverse primers and for the Taqman probes. The probes were synthesized with a 5' FAM reporter dye and a 3' TAMRA quencher. The primers and probes had a Tm of 60 and 70°C, respectively. Shown are also the optimal concentrations in the PCR, the PCR efficiencies and the $X_{0,target}/X_{0,ref}$ values of the genes in naive DRGs.

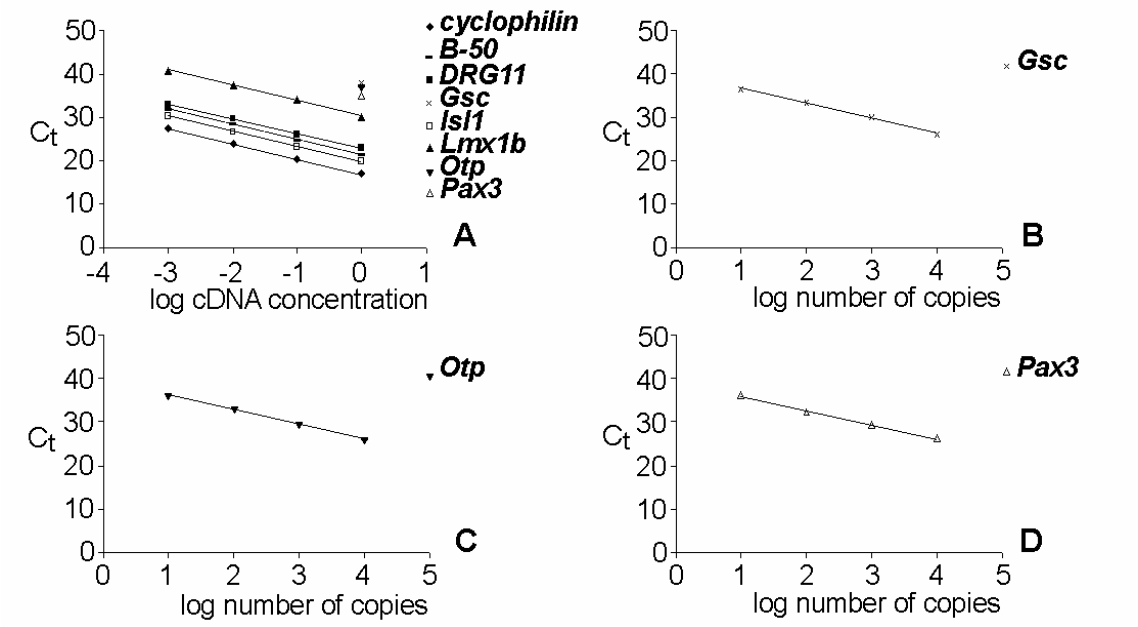

Figure 1: Validation of the quantitative PCR
(A) Standard curves plotting the C_t values against the logarithm of the cDNA concentration for *cyclophilin*, *B-50*, *DRG11*, *Isl1*, and *Lmx1b*. (B,C,D) Standard curves of *Gsc*, *Otp*, and *Pax3* assessed on dilution series of copies of the PCR products.

Quantification of the homeobox genes in mouse DRGs after nerve crush

There were substantial differences in expression levels of the homeobox genes in naive DRGs. *DRG11* and *Isl1* were the most highly expressed ($X_{0,target}/X_{0,ref}$ around 0.03, corresponding to levels about 30 times less than *cyclophilin*); *Gsc*, *Otp*, and *Pax3* were present at very low levels ($X_{0,target}/X_{0,ref}$ around 1E-06, corresponding to levels about 1,000,000 times lower than *cyclophilin*) (Table 2).

Figure 2 shows the expression levels of *B*-50, *Isl1*, *DRG11*, *Lmx1b*, and *Pax3* relative to the sham-operated DRGs at 1, 4 and 7 post-operative days (dpo). In general, expression levels in the ipsilateral crush-lesioned DRGs were regarded as different only if they differed significantly from each of the other samples at the same time point. It has been well established that the growth-associated protein B-50 (or GAP-43) is upregulated after crush. Therefore, *B-50* was used as positive controls of the regeneration model. At 1 dpo, *B-50* was not significantly changed, whereas at 4 and 7 dpo, the relative *B-50* levels were increased in crush-lesioned ipsilateral DRGs (one way ANOVA, with posthoc LSD 0.001 and 0.020, respectively (LSD = least significant difference)). The levels at 4 dpo were increased 3.8-fold as compared to the calibrator. At 7 dpo the relative *B-50* levels were more variable, the highest upregulation being 8.0-fold, the lowest 2.8-fold; on average *B-50* was 5.4-fold increased. The increased expression levels of *B-50* were confirmed with *in situ* hybridization (Figure 3). At 1 dpo *Isl1* showed a significant 2.8-fold decrease in crush-lesioned ipsilateral DRGs (one way ANOVA, with posthoc LSD 0.002) relative to the sham-operated contralateral DRGs (calibrator). At 4 and 7 dpo, the relative levels of *Isl1* were not significantly different between experimental groups.

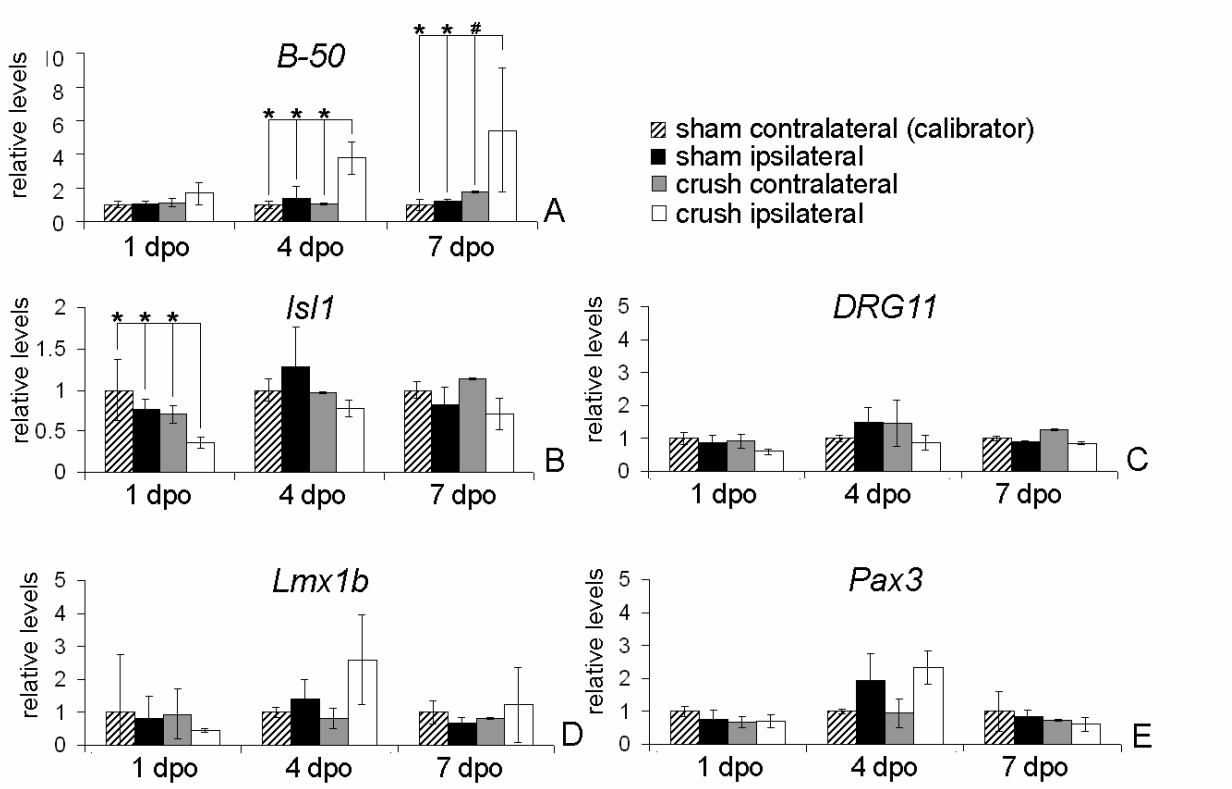

Figure 2: Quantification of homeobox genes in mouse DRGs during regeneration
Quantitative PCR of the growth-associated protein *B-50* (A) and the homeobox genes *Isl1* (B), *DRG11* (C), *Lmx1b* (D), and *Pax3* (E) in sham- and crush-operated contra- and ipsilateral DRGs at 1, 4 and 7 dpo. Each bar represents the average of three samples of three pooled mice per experimental group. Shown are the expression levels relative to the sham-operated contralateral DRGs (hatched).
* = significant, $p<0.05$; # = borderline significant, $p=0.053$

No significant differences in *DRG11* and *Pax3* expression levels were observed between ipsi- and contralateral sham- and crush-operated DRGs at all time points (Figure 2). *Lmx1b* was expressed at intermediate levels in the DRGs and did not differ significantly between experimental groups (Figure 2). *Gsc* and *Otp* were expressed at more variable levels. The $X_{0,target}/X_{0,ref}$ values for these genes varied from ~6E-04 to ~1E-07 between and within experimental groups. These values correspond to a difference in expression level of about 6,000-fold between the highest and lowest expressing samples. Because of this variation, no expression levels relative to the calibrator were calculated for these genes.

DISCUSSION

Based on the hypothesis that molecular mechanisms underlying regeneration might recapitulate on developmental processes, we screened for alterations in homeobox gene expression after sciatic nerve crush in the rat DRGs. The selected genes *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp*, and *Pax3* were quantified in the mouse using real-time quantitative PCR. *Vsx2-like* could not be quantified because of the lack of sequence information to select primers on. This gene is not contented in the available databases. We used the mouse sciatic nerve crush model, because of the availability of mouse homeobox gene sequences in the databases and the potential to use mouse mutants as

experimental animal in regeneration experiments [Cafferty et al, 2001; Siconolfi and Seeds, 2001; Zhong et al, 1999]. We investigated the validity of this model before, showing that the mice displayed normal regeneration-associated gene expression and normal recovery of sensory and motor function [Vogelaar et al, *submitted*]. Here we showed that homeobox genes expressed at extremely low levels, about 1,000,000 times lower than *cyclophilin*, could be quantified accurately, and we confirmed that *B-50* was upregulated in the crush-lesioned mouse DRGs, consistent with the known upregulation in rats [Hol et al, 1999; Plantinga et al, 1993a; van der Zee et al, 1989; Woolf, 1990; Zhang, 2000]. Judging from this response of *B-50* expression and from the functional recovery [Vogelaar et al, *submitted*] of the mice, we concluded that the regeneration-model in the mice was appropriate to measure alterations in homeobox gene expression.

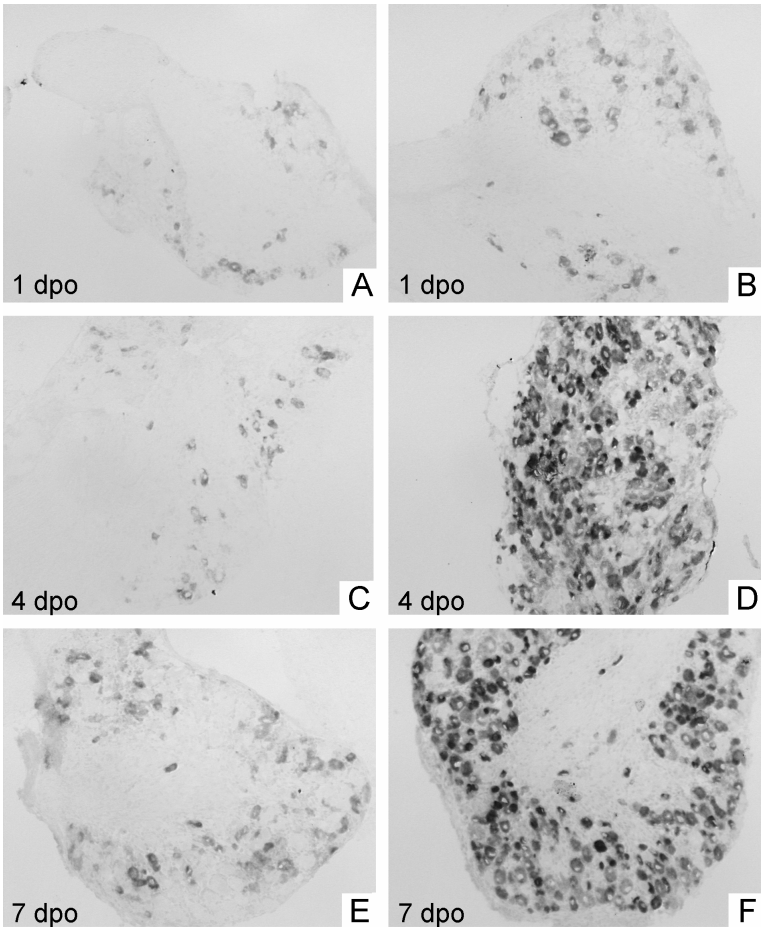

Figure 3: *B-50* expression in regenerating mouse DRGs
In situ hybridization with *B-50* probe on contralateral (left column) and ipsi-lateral (right column) DRGs, showing that *B-50* is expressed at higher levels and in a larger number of neurons at 4 and 7 dpo.

The regeneration-associated changes in expression of the homeobox genes studied here appeared to be very selective. First of al, *Gsc* and *Otp* displayed highly variable levels within and between all experimental groups. This variability in levels was unlikely to be due to intrinsic variability of the PCR, because of the extensive validation that preceded the experiment. Selective loss of mRNA copies during the RNA isolation procedure was also not likely, judging from the measurements of *Pax3*, which was expressed consistently at extremely low levels. We think that the variation in expression levels of *Gsc* and *Otp* is a biological phenomenon possibly related to a variable expression level or number of cells expressing these genes. There may exist a small subpopulation of neurons expressing high levels of *Gcs* and *Otp*, distributed variably over different DRG levels.

We detected a 2.8-fold decrease in *Isl1* expression levels exclusively at 1 dpo, indicating a transient effect of the crush lesion on the expression of this homeobox gene. Hol et al (1999) reported a weak downregulation at 7 dpo using semi-quantitative RT-PCR in rats. This may reflect a fast return of *Isl1* expression to normal levels, which is in accord with our findings. The function of *Isl1* in developing DRGs, to date, is not known, although it is abundantly expressed in many DRG neurons during development. In the spinal cord, *Isl1* importantly contributes to the combinatorial genetic network that controls motor neuron development [Shirasaki and Pfaff, 2002; Tanabe and Jessell, 1996]. Moreover, it has been indicated that *Isl1* is involved in outgrowth, pathfinding and neuroendocrine phenotypes of neurons [Thor et al, 1991; Thor and Thomas, 1997]. The downregulation of *Isl1* may be associated with the downregulation of the neurotransmitter expression during regeneration.

In contrast to the change in *Isl1* expression, we found that *DRG11* and *Pax3* expression levels were not different between experimental groups. This was unexpected, as both of these genes are key players in DRG development. *DRG11* is expressed in many (if not all) embryonic DRG neurons and is known to remain expressed in the adult DRGs, in a restricted subpopulation of neurons [Saito et al, 1995, Vogelaar et al, *in press*]. The *DRG11* knock-out mouse revealed that *DRG11* is important for the establishment of connections between DRG neurons and their targets, and for the maintenance of adult neurons [Chen et al, 2001]. A recapitulation of developmental processes during regeneration would suggest a role for *DRG11* either in survival of the neurons or in outgrowth/pathfinding after crush. However, *DRG11* expression was not upregulated after nerve crush, as would be expected from its embryonic expression patterns.
Pax3 is involved in early migration of the neural crest cells, in Schwann cell development and in DRG neuron differentiation [Anderson, 1999; Goulding et al, 1991; Koblar et al 1999]. Moreover, *Pax3* induction during regeneration has been reported in the Schwann cells at the distal nerve stump during regeneration. However, we show here that in the DRG itself, *Pax3* does not display alterations in expression levels after crush. This indicates that *Pax3*, present in developing DRG neurons [Goulding et al, 1991], is not reinduced in regenerating DRG neurons. These data indicate that, in contrast to Schwann cells, DRG neurons do not react to injury simply by dedifferentiation. The neurons retain their adult expression levels of *DRG11* and *Pax3*.
Finally, *Lmx1b* also points into the direction of persistence of adult homeobox gene expression patterns in regenerating neurons. Examination of *Lmx1b* expression in embryonic DRGs revealed that this gene is not expressed during development of the DRG [Asbreuk et al, 2002b]. Notably, adult DRG neurons do express *Lmx1b* [Vogelaar et al, *in press*]. A recapitulation of development, therefore, would be effectuated in the downregulation of *Lmx1b*, which, as shown here, does not occur.

We conclude that the developmental expression patterns of the homeobox genes studied here are not recapitulated during regeneration, indicating that the hypothesis that developmental processes might play a role in regeneration of DRG neurons should be reconsidered. Our data indicate that gene expression programs controlled by these homeobox genes during development are not directly involved in sciatic nerve regeneration, but rather point to regeneration-specific mechanisms.

ACKNOWLEDGEMENTS

We would like to thank Marije Rensen-de Leeuw for providing us with the *B-50* probe and Elly Hol for fruitful discussions on the interpretation of the quantitative measurements and for carefully reading the manuscript of this chapter.

Chapter 5

Differential function of *Gsc* in the development of neural crest derivatives:

Dorsal root ganglion neurons do not require *Gsc*

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submitted

ABSTRACT

Goosecoid (*Gsc*) is a paired-like homeobox gene expressed in the early embryo during gastrulation and at later stages during organogenesis. It is involved in the development of craniofacial mesectodermal structures, which are derivatives of the neural crest. In a previous study on homeobox gene expression in adult dorsal root ganglia (DRGs) we found *Gsc* expression. As dorsal root ganglia are neural crest derivatives, we investigated if the *Gsc* null mutation affected the expression of neurotrophin receptors in mouse DRGs, immediately after birth. Since *Gsc* may be part of a transcription factor cascade in DRGs, we also investigated the expression of the homeobox genes *Brn3a*, *Brn3b*, *DRG11*, *Isl1*, *Oct1*, and *Prx3*. No difference was observed between wild type and null mutant animals in expression and distribution of the genes studied. We conclude that *Gsc* does not play a predominant role in the development of the major subpopulations of DRG neurons and that *Gsc* is not involved in the regulation of expression of the other homeobox genes studied in the DRGs. This contrasts the role of *Gsc* in other neural crest derivatives, like craniofacial mesectodermal structures, and points to tissue-specific functions of *Gsc* in neural crest differentiation.

INTRODUCTION

Goosecoid (*Gsc*) is a paired-like homeobox gene originally identified in the initial stages of development, specifically in the organizer region and the primitive streak responsible for gastrulation. It has been implicated in the formation of axial structures, e.g. somites and the neural tube [Blum et al, 1992; Niehrs et al, 1993]. *Gsc* is also expressed during organogenesis (from day 10.5 onwards) in branchial arch 1 and in the anterior one-third of branchial arch 2 [Gaunt et al, 1993]. Tissues derived from these arches are specific structures in the head, the limbs and the ventrolateral body wall. *Gsc* null mutants do not display abnormalities in gastrulation but die soon after birth because of multiple craniofacial defects, including defects in the lower mandible, palatine, nasal cavity, nasal capsules and turbinals. The nasal septum fails to fuse with the palate. The orbital processes of the maxillary and frontal bones that support the eye are reduced. Several structures in the ear are also affected [Belo et al, 1998; Yamada et al, 1995].

These structures, affected by the *Gsc* null mutation, are all mesectodermal derivatives of the neural crest, a highly plastic structure that arises at the lateral edges of the neural plate [Belo et al, 1998; Gaunt et al, 1993; Le Douarin and Kalcheim, 1999; Yamada et al, 1995]. During the process of neurulation, the neural plate folds to give rise to the neural tube. Neural crest cells detach and migrate to multiple places in the body where they form many tissue types including mesectodermal structures of the head, neurons and glia in the sensory ganglia (among others dorsal root ganglia), sympathetic ganglia and the enteric nervous system. [Le Douarin and Kalcheim, 1999; Hall, 1999]. The data from the *Gsc* null mutant mice suggest that *Gsc* is highly important for the development of several craniofacial neural crest derivatives [Belo et al, 1998; Le Douarin and Kalcheim, 1999; Yamada et al, 1995].

Previously, we identified *Gsc* amongst the homeobox genes expressed in the dorsal root ganglia (DRGs) of adult rats [Vogelaar et al, *in press*]. Based upon this observation and based upon the multiple abnormalities in neural crest derivatives in the *Gsc* null mutant mice, we hypothesized that DRG development would be affected in the *Gsc* null mutant. We determined the expression of the neurotrophin receptors, *TrkA*, *TrkB*, *TrkC*, and *cRET*, in order to investigate the distribution and abundance of subpopulations of DRG neurons in the *Gsc* null mice. Furthermore, we determined expression of *B-50* (also known as *GAP-43*), a growth-associated protein [Oestreicher

et al, 1997] and of two neuropeptides, *galanin* and *cholecystokinin* (*CCK*), to discriminate smaller neuronal subpopulations [Bergman et al, 1999; Hökfelt et al, 1994]. Since *Gsc* may be part of a transcription factor cascade in DRGs, we also included other homeobox genes expressed in developing and adult DRGs. No difference was observed in DRGs between wild type and null mutant animals in expression and distribution of the genes studied, pointing to a tissue-specific role of *Gsc* in the development of different neural crest derivatives.

EXPERIMENTAL PROCEDURES

Breeding and genotyping

The animal procedures were performed in accordance with the Ethical Committee on Animal Experiments of the University of Utrecht. *Gsc* heterozygous mice (C57BL/6J background) were obtained from the European mouse mutant archive (EMMA, Italy) with permission of Prof. P. Gruss, whose group originally made the knock-out [Yamada et al, 1995]. P0 newborn pups were killed by decapitation and frozen on dry ice. Transverse cryostat sections of 16 µm were cut at trunk levels to perform hematoxylin and eosin (HE) staining (following standard procedures) and *in situ* hybridization. For genotyping, DNA was prepared from tail biopsies of the newborn mice. Genotyping was performed using primers described by Belo et al (1998).

Real-time quantitative PCR

For quantitative analysis DRGs were isolated from P0 null mutant, heterozygote and wild type *Gsc* mice. Briefly, the spinal column was dissected and cleaned from surrounding tissue. The spinal column was opened longitudinally along the back axis and the spinal cord was removed using a P100 pipet. DRGs were isolated using sharp tweezers, pooled and frozen in an Eppendorf vial in dry ice. Total RNA was extracted from the DRGs following the TRIzol isolation method (Life Technologies) according to the manufacturer's protocol. To ensure purity of the RNA an additional chloroform extraction step was performed.
Directly after RNA isolation, first strand cDNA synthesis was performed in a final volume of 50 µl containing 1x first strand buffer, 0.01 M DTT, 750 ng random hexamers, 250 U Superscript II RNase H- reverse transcriptase, 500 µM dNTPs and 36 U RNAguard. Primers and fluorogenic probes for *cyclophilin* and *Gsc* (Table 1) were used as described previously [Bogerd et al, 2001]. In short, optimal primer/probe concentrations were determined using 10^4 copies of cloned PCR product and PCR efficiencies were measured with tenfold dilution series of these copies (Table 1). Quantitative measurements were performed in triplicate on 5 µl of cDNA during 50 cycles. The ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used and data were collected using the Sequence Detection Software provided by the manufacturer. For every sample, an amplification plot was generated, showing the increase in the reporter dye fluorescence with each PCR cycle. The reporter signal was normalized to the fluorescence of an internal reference dye. From each amplification plot, a threshold cycle (C_t) value was determined, representing the PCR cycle number in the exponential phase of the PCR [Bustin, 2000]. The C_t value is inversely proportional to the log of the initial mRNA copy number.
To correct for differences in cDNA load between the samples, the *Gsc* PCR was normalized to *cyclophilin*, a housekeeping gene often used in quantitative studies [Medhurst et al, 2000]. The expression levels of *Gsc* ($X_{0,Gsc}$) relative to *cyclophilin* ($X_{0,Cyclo}$) were calculated using the equation $X_{0,Gsc}/X_{0,Cyclo} = E^{-Ct,Gsc}/E^{-Ct,Cyclo}$ in which E is the PCR efficiency of the individual genes (Table 1) as described by Kamphuis et al (2001).

Table 1: Sequence, positions, and properties of primers and probes used for quantitative PCR

Gene	Accession number	Primer	positions	Sequence 5'- 3'	Optimal conc. (nM)	PCR Efficiency
cyclophilin	NM_008907.1	forward	19-35	GCTTTTCGCCGCTTGCT	300	1,91
		reverse	86-69	CTCGTCATCGGCCGTGAT	300	
		probe	43-66	TGGTCAACCCCACCGTGTTCTTCG	200	
Gsc	NM_010351.1	forward	939-962	TTGCACAGACAGTCGATGCTACTT	900	1,93
		reverse	1037-1014	TCCTGGGCCTGTACATTATTTACA	900	
		probe	984-964	CACACACCCTGCCTTGCGGGA	200	

Optimal primer/probe concentrations were determined on 10^4 copies of cloned PCR products. Efficiency measurements were done on a dilution series of these copies.

Probes and in situ hybridization

Digoxygenin-labeled RNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Antisense and sense probes were used for the markers *TrkA* (derived from M85214), *TrkB* (derived from NM_012731), *TrkC* (derived from S60953), *cRET* (derived from X67812), *B-50**, *galanin* [Vrontakis et al, 1987], and *CCK* [Deschenes, 1984] and for the homeobox genes encoding *Brn3a* and *-3b* [Theil et al, 1994], *DRG11**, *Gsc**, *Lmx1b* [Chen et al, 1998], and *Prx3* [van Schaick et al, 1997]. The probe length varied from about 300 to 1300 bp. Probes marked with an asterisk are obtained from cloned PCR products or cDNAs available at our own facilities. In situ hybridization was carried out on 16 µm cryostat sections as described before [Vogelaar et al, *in press*].

RESULTS

Quantification of Gsc in DRGs of newborn mice

We performed quantitative PCR in order to check whether *Gsc* was expressed in DRGs of newborn P0 mice. We found *Gsc* expression in wild type as well as heterozygote and homozygote null mutant DRGs, at equal levels. The mean $X_{0,Gsc}/X_{0,Cyclo}$ was 1.7E-03, indicating that *Gsc* is expressed about 590 times lower than *cyclophilin*, a housekeeping gene. However, *Gsc* levels were too low to be made visible with *in situ* hybridization (data not shown).

Histological analysis and *in situ* hybridization with DRG neuronal markers

Hematoxilin eosin (HE) staining of transverse sections at trunk levels of P0 *Gsc* null mutant mice showed normal location and size of the DRGs (Figure 1). We performed *in situ* hybridization with the neurotrophin receptors, *TrkA*, *TrkB*, *TrkC*, and *cRET*, to investigate the distribution of the DRG neuronal subpopulations. *TrkA* was expressed in many small-sized neurons and was equally distributed over the wild type and null mutant DRGs. *TrkB* and *TrkC* marked medium- to large-sized neurons and were also unaffected by the *Gsc* null mutation. *cRET* was expressed in many neurons of both wild type and null mutant DRGs (Figure 2).The expression patterns of three other markers, *galanin*, expressed at low levels in a subpopulation of neurons; *CCK*, expressed in a very small number of neurons; and the growth-associated protein *B-50*, were also normal (Figure 2).

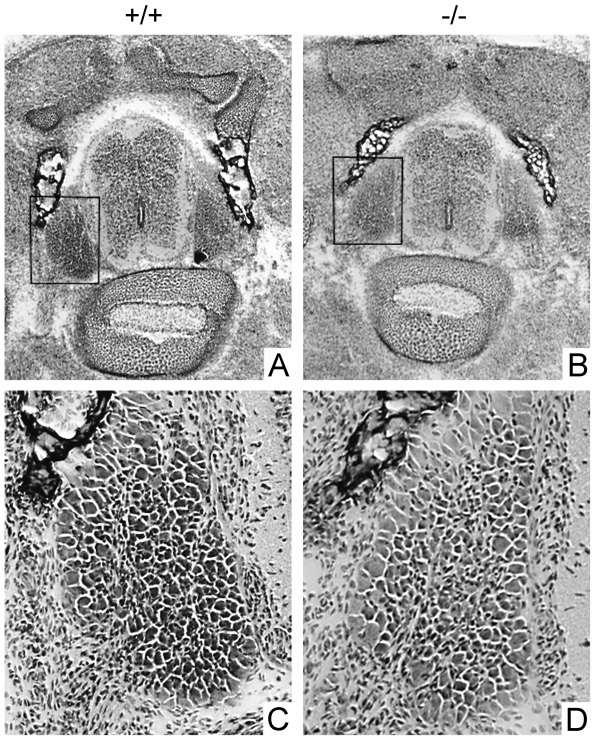


Figure 1: Histological analysis of *Gsc* null mutant P0 DRG
Hematoxylin eosin staining of transverse sections through the spinal column and DRGs at trunk levels of wild type (A,C) and null mutant (B,D) *Gsc* mice. Boxed DRGs in A and B are enlarged in C and D. No differences in DRG size and location were observed.

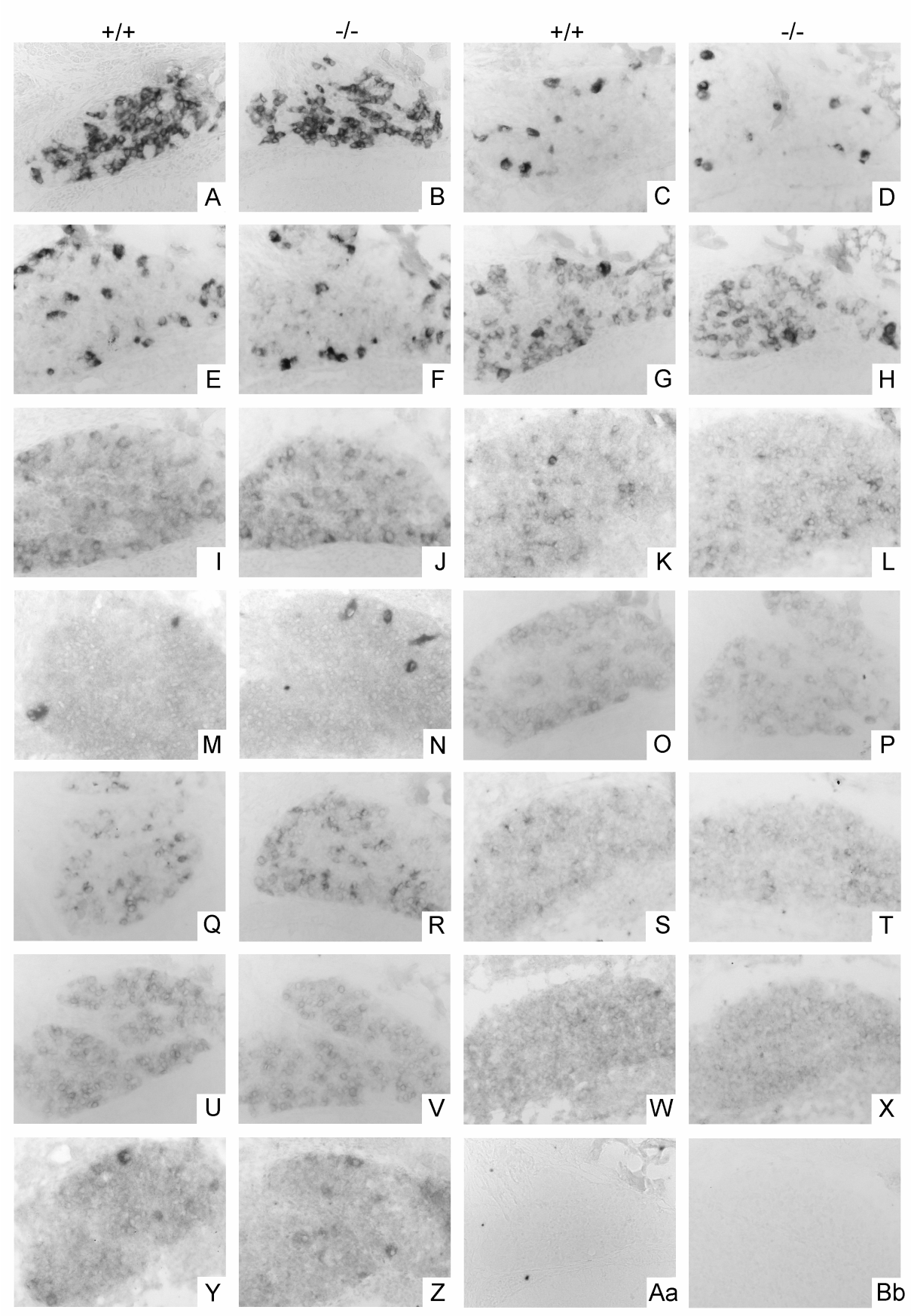
Expression of other homeobox genes in *Gsc* null mutant DRGs

In order to investigate whether *Gsc* would be involved in cascades of homeobox genes during development, we performed *in situ* hybridization with probes for *Brn3a*, *Brn3b*, *DRG11*, *Isl1*, *Oct1*, and *Prx3*. In general, these homeobox genes were expressed at much lower levels than the other markers (Figure 2). In the P0 *Gsc* null mutant and wild type mice *Brn3a*, *DRG11*, and *Isl1* lightly stained many if not all DRG neurons. *Oct1* was expressed ubiquitously in all embryonic tissues, including DRGs. *Brn3b* was expressed in small neurons, whereas *Prx3* expression was highest in large, and low in small DRG neurons. There was no difference in the expression of these homeobox genes between wild type and mutant mice (Figure 2).

DISCUSSION

Based upon the previous observation of *Gsc* expression in adult DRGs, and based upon the known involvement of *Gsc* in development of neural crest derivatives, we hypothesized that *Gsc* may be involved in development of DRGs. Using quantitative PCR, we confirmed expression of *Gsc* mRNA in the DRGs of P0 newborn mice. The *Gsc* PCR was positive in wild type as well as heterozygote and null mutant DRGs, because the primers were located in a region of the gene that was not deleted by the *Neo* insert. HE staining of *Gsc* null mutant DRGs showed normal location and size, indicating no major role for *Gsc* in early developmental processes, like neural crest migration, proliferation, and survival of DRG neurons.

Next page:
Figure 2: Distribution of DRG neuronal markers and homeobox genes
In situ hybridization on P0 DRGs from wild type mice (first and third column) and *Gsc* null mutant mice (second and fourth column) with *TrkA* (A,B); *TrkB* (C,D); *TrkC* (E,F); *cRET* (G,H); *B-50* (I,J); *galanin* (K,L); *CCK* (M,N); *Brn3a* (O,P); *Brn3b* (Q,R); *DRG11* (S,T); *Isl1* (U,V); *Oct1* (W,X); and *Prx3* (Y,Z). Control stainings with a sense probe are shown in Aa and Bb.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The expression patterns of the neurotrophin receptors were used to discriminate between the different neuronal subpopulations in the DRGs. *TrkA*, the high affinity NGF receptor, is a marker for developing nociceptive and thermoceptive neurons. *TrkC*, the high affinity receptor for NT-3, stains future proprioceptive neurons. *TrkB*, the BDNF receptor, marks mechanosensory DRG neurons [Bibel and Barde, 2000] and *cRET* is expressed in GDNF-dependent neurons [Molliver et al, 1997]. No differences in the distribution and abundance of neurons positive for these markers were observed, indicating that roughly, all subpopulations of DRG neurons are present. To look more closely to smaller subpopulations of neurons, we stained with *galanin* and *CCK*, two genes involved in nociception [Bergman et al, 1999; Hökfelt et al, 1994; Xu et al, 1996], but these also showed no difference between wild type and null mutant DRGs. Expression of the growth-associated protein *B-50* [Oestreicher et al, 1997] was normal, indicating that the mechanisms for outgrowth in these animals were probably intact.

Since *Gsc* may be part of a transcription factor cascade in DRGs, we included other homeobox genes expressed in DRG development. *Brn3a* and *Brn3b* are known factors in embryonic as well as adult DRGs [Akopian et al, 1996; Xiang et al, 1995]. *Brn3a* is essential for outgrowth and survival of sensory neurons during DRG development [Eng et al, 2001]. *DRG11* is required for the formation of projections from nociceptive neurons to their central targets and for the survival of neurons after birth [Chen et al, 2001]. *Isl1* remains expressed in adult DRGs and is thought to be important for maintenance of the neuroendocrine phenotype [Thor et al, 1991; Hol et al, 1999]. Finally, *Oct1* and *Prx3*, found in a previous study in adult DRGs [Vogelaar et al, *in press*] were also included in this study. No difference in the expression of these homeobox genes was observed between wild type and mutant mice, indicating that the expression of these transcription factors does not require *Gsc*.

We show here that the *Gsc* null mutant mice do not have a phenotype in DRG location, size, neuronal differentiation and transcription factor expression. These results are remarkable, since *Gsc* has been shown to be essential for the development of other neural crest derived tissues, like craniofacial mesectodermal structures [Belo et al, 1998; Le Douarin and Kalcheim, 1999; Yamada et al, 1995]. It may be that cells with the same origin, in this case the neural crest, retain low levels of genes originally expressed. We conclude that *Gsc* does not play a predominant role in the development of the major subpopulations of DRG neurons and that *Gsc* is not involved in the regulation of expression of the other homeobox genes studied in the DRGs. These data point to a tissue-specific role of *Gsc* in the development of different neural crest derivatives.

ACKNOWLEDGEMENTS

We would like to thank Marije Rensen-de Leeuw for providing us with the *B-50* probe and Johan Miedema for his useful essay on the DRG neuron markers. We are very grateful to Jan Bogerd for the use of the ABI Prism 7700 equipment and for his help in setting up the real-time qPCR technique.

Chapter 6

Overview and general discussion

OVERVIEW

In the past years many groups have investigated alterations in expression of genes after lesioning the sciatic nerve. Cytoskeletal proteins, neurotransmitters (and/or neurotransmitter enzymes), adhesion molecules, growth factors, cytokines, transcription factors and many more protein families undergo transcriptional alterations after nerve damage (summarized in Table 1). These proteins are thought to be functionally involved in regeneration processes and many participate in the development of the peripheral nervous system. Therefore, it has been proposed that developmental processes are recapitulated during regeneration. However, there are also reports indicating a lack of developmental recapitulation or a difference in gene expression between the regenerating and developing nerves. In this thesis, we aimed to use homeobox gene expression as a tool to address the question whether the molecular mechanisms of DRG neuron regeneration recapitulate developmental mechanisms.

The studies were initiated with a PCR-based screen for homeobox gene expression in the adult rat DRGs in order to make an inventory of the homeobox gene repertoire used by this tissue **(chapter 2)**. Twenty-two homeobox genes were identified of which six were demonstrated to be expressed in DRG neurons by *in situ* hybridization. This also revealed the major "problem" in analyzing homeobox gene expression: their low abundance. The current methods used for visualizing mRNAs or proteins in tissue sections seem not to be sufficiently sensitive to detect all homeobox genes in the DRGs. In order to be able to analyze in more detail homeobox gene expression during regeneration, therefore, we were limited to PCR-based methods. Therefore, we think that the current knowledge about the expression of transcription factors in the DRGs may be far from complete. More sensitive histological methods, like *in situ* PCR or single cell expression analysis currently being developed might be very useful to overcome these problems.

For studies on the regulation of selected homeobox genes we used the mouse as experimental animal, which provides the opportunity to relate functional aspects to expressed homeobox genes due to the availability of genetically modified mice. We used the C57BL/6J mouse strain, often used as background of knock-out mice. **Chapter 3** describes the characterization of sciatic nerve regeneration in this mouse strain, both at the functional and at the molecular level. We showed that the injured mice displayed normal recovery of sensory and motor functions, similar to that observed in rats. No obvious loss of specific neuronal subpopulations was observed, and alterations in gene expression after crush were similar to those occurring in other mouse strains or in rats. From chapter 3 we concluded that the C57BL/6J mouse is a useful strain to monitor regeneration-related alterations in gene expression after sciatic nerve crush.

Chapter 4 describes the quantitative analysis of several homeobox genes at three time points after crush. We confirmed that *Isl1* decreased after sciatic nerve injury. Since *Isl1* is expressed at high levels in many neurons during DRG development, it was concluded that embryonic *Isl1* expression patterns were not recapitulated during development. *Pax3* and *DRG11*, two homeobox genes with well-characterized functions in DRG development, did not change in expression levels after nerve crush, whereas an upregulation would be expected if embryonic expression would have been recapitulated. Furthermore, the expression levels of *Lmx1b*, a homeobox gene that is expressed in the adult but not in the embryonic DRGs, were also not altered; a recapitulation of development would be reflected by a decreased expression of this gene. Finally, *Gsc* and *Otp* showed highly variable expression levels, due to which no conclusion about involvement of these two homeobox genes in regeneration could be made. Expression of *Gsc* and *Otp* has not been described in DRGs before. We hypothesized that *Gsc*, being essential for the

development of other neural crest derivatives, may be involved in DRG development as well. However, in **chapter 5** we showed that the major subpopulations of DRG neurons are present in neonatal *Gsc* null mutant mice. Furthermore, in DRG development, *Gsc* was not involved in the regulation of expression of the other homeobox genes studied. From chapter 5 we concluded that *Gsc* is differentially involved in the development of different neural crest derivatives.

GENERAL DISCUSSION:

IS REGENERATION A RECAPITULATION OF DEVELOPMENT?

From the observations described in this thesis we conclude that the developmental expression patterns of the homeobox genes are not just recapitulated during regeneration of adult DRG neurons. Therefore, the hypothesis that developmental processes might play a role in regeneration of DRG neurons will be reconsidered and discussed below in more detail.

Homeobox genes and the mechanisms of regeneration

The regulation of transcription factor expression in adult DRG neurons is likely to underlie the molecular mechanisms of regeneration-associated gene expression alterations in injured neurons. These molecular mechanisms were hypothesized to be recapitulating developmental processes, as expected from the many developmentally expressed genes that are reexpressed after injury. Homeobox genes were likely candidates for investigating the recapitulation of development, because of their functions in neural development and their involvement in the Schwann cell reaction to injury.
Our conclusion that regenerating DRG neurons do not just recapitulate their developmental expression patterns has been based on the quantitative analysis of four homeobox genes, *DRG11*, *Isl1*, *Lmx1b* and *Pax3*. *DRG11* and *Pax3* have known functions in DRG neuronal differentiation, outgrowth and survival [Chen et al, 2001; Koblar et al, 1999; Patapoutian, 2001; Saito et al, 1995], and *Isl1* is likely to be involved in processes like outgrowth, pathfinding and neuroendocrine phenotype [Hol et al, 1999; Thor et al, 1991]. In line with our experiments the POU homeobox gene, *Brn3a*, known to be involved in embryonic DRG outgrowth and survival [Eng et al, 2001], was reported to be downregulated after sciatic nerve injury [Begbie et al, 1996; Küry et al, 2001a], suggesting that *Brn3a*, too, does not have the same function in regenerating and developing DRGs. *Oct2* was reported to be slightly upregulated after crush [Begbie et al, 1996], which could reflect both adult and embryonic mechanisms, as it is highly expressed in both embryonic and adult DRGs [Begbie et al, 1996].

From the lack of a recapitulation of developmental homeobox gene expression it follows that the transcriptional mechanisms for DRG neuron differentiation, outgrowth and survival are different between regenerating and developing DRG neurons. This indicates that, in contrast to Schwann cells, the reaction of DRG neurons to injury is not simply a dedifferentiation followed by redifferentiation. The phenotype adopted by the injured neurons to initiate regeneration, therefore, is not an embryonic phenotype, but reflects adult mechanisms for outgrowth and pathfinding. This can be considered as a regeneration-specific mechanism reflected by specific changes in gene expression, summarized in Table 1. This may not be surprising since the cellular environment and the connectivity of the neurons is profoundly different in the adult versus the embryo. DRG neurons develop in the vicinity of their developing targets, i.e. dermamyotome, which develops from differentiating somites. Thus, the distance traveled and the access to target-derived factors are dramatically different between development and regeneration. The axonal

environment is also fundamentally different due to cytokines produced by invading macrophages and Schwann cells (Table 1). The way external signaling molecules participate in the response of injured DRG neurons may be an important aspect of the regeneration-specific mechanism. In the following section, signaling and transcription processes relevant for the molecular mechanisms of regeneration will be addressed.

Signal transduction pathways used during regenerative outgrowth

The mechanisms underlying regenerative growth are likely to involve neurotrophic factors. The decrease in availability of NGF, due to the disruption of the nerve fibers and the decrease in the rate of retrograde transport [Lee et al, 1998; Raivich and Kreutzberg, 1993], is generally regarded as the main trigger for the response of DRG neurons to injury [Aldskogius et al, 1992; Fawcett and Keynes, 1990; Verge et al, 1996]. This is supported by Shadiack et al (2001) showing induction of axotomy-like changes in neuropeptide expression in DRG neurons after the application of NGF antiserum. It seems likely that the upregulation of neurotrophins by Schwann cells and satellite cells (Table 1) observed after injury play important roles in the mechanisms underlying regeneration. However, as described in chapter 3 of this thesis, the Trk receptors (TrkA, -B, and -C) on DRG neurons are not induced. *TrkA* is even downregulated, indicating that developmental Trk expression patterns are not recapitulated (Table 1). Moreover, the signal transduction pathways used by DRG neurons during development and regeneration have been recently been reported to be different. Extracellular signal-related kinase (ERK) and phosphatidylinositol-3 kinase (PI3-K), the major signal transduction pathways activated by the Trk receptors [for review, see Kaplan and Miller, 2000], were shown to be involved in axonal outgrowth from embryonic DRGs, but not from lesioned DRGs [Liu and Snider, 2001]. ERK phosphorylation after injury has been reported in Schwann cells, but not in DRG neurons [Abe et al, 2001; Sheu et al, 2000].

If neurotrophins are not directly involved in regenerative outgrowth, what proteins then are? Although their role in inflammatory responses is well known, cytokines also have effects on the nervous system. The so-called neuroactive cytokines include ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), fibroblast growth factor (FGF) and transforming growth factor (TGF) [Gadient and Otten, 1997; Grothe and Nikkhah, 2001; Murphy et al, 1997, Unsicker et al, 1992]. Of these, only CNTF expression has been reported to decrease after nerve injury, the others are all upregulated (Table1) [for review, see Murphy et al, 1997; Markus et al, 2002]. Neuroactive cytokines signal through gp130 receptors upstream of signaling molecules like JAK2 and STAT3. In contrast to the apparent disuse of neurotrophin signaling in regenerative outgrowth, Liu and Snider (2001) observed that JAK2 and STAT3 are involved in regenerative outgrowth from lesioned DRGs, but have no effect on embryonic DRGs. Moreover, the transcription factor STAT3 is phosphorylated in DRG neurons after nerve injury [Liu and Snider, 2001].

These data indicate that cytokines may be important in controlling regeneration-associated gene expression in DRG neurons through the JAK/STAT signal transduction pathway [Markus et al, 2002]. In line with this, genes known to be induced by LIF and IL-6, *galanin*, *peripherin*, and *Reg-2* [Cafferty et al, 2001; Livesey et al, 1997], are upregulated in DRG neurons (Table 1) [Averill et al, 2002; Villar et al, 1989; Wong and Oblinger, 1990]. Genes known to be induced by NGF, among others *SP* and *CGRP* [Lindsay and Harmar, 1989; Mohiuddin et al, 1999; Verge et al, 1995], are downregulated in response to nerve injury (Table 1) [Henken et al, 1990; Hökfelt et al, 1994; Villar et al, 1989].

Moreover, LIF null mutant mice have no phenotype in the development of DRG neurons, but in the adult null mutants sciatic nerve regeneration is impaired and small peptidergic neurons die after injury [Cafferty et al, 2001].

The mechanisms of cell survival may also differ between regenerating and developing neurons. The upregulation in DRG neurons of both neuroactive cytokines, like TGFβ and basic FGF, and their receptors suggest autocrine mechanisms for survival after injury. NGF and BDNF are also upregulated in DRG neurons after nerve injury [Sebert and Shooter, 1993] (also pointing to an autocrine loop), but as mentioned before their receptors are not, indicating again a less important role for neurotrophins. The dependency for survival on target-derived neurotrophic support seems to be absent in adult and regenerating neurons [Lindsay, 1992].

These above data corroborate the notion that the molecular mechanisms underlying gene expression alterations during regeneration are different from those used in development. Observations point to a regeneration-associated role of cytokines, rather than neurotrophins, in outgrowth and survival of DRG neurons (Figure 1) [Markus et al, 2002]. However, it is important to note that this does not rule out a function for neurotrophins in regeneration, but that it does contradict a direct effect of neurotrophins on DRG neurons. The enhanced production of endogenous neurotrophins by Schwann cells, may not have direct effects on DRG neurons, but are thought to indirectly stimulate regeneration through effects on Schwann cells [Mohiuddin et al, 1999] (Figure 1).

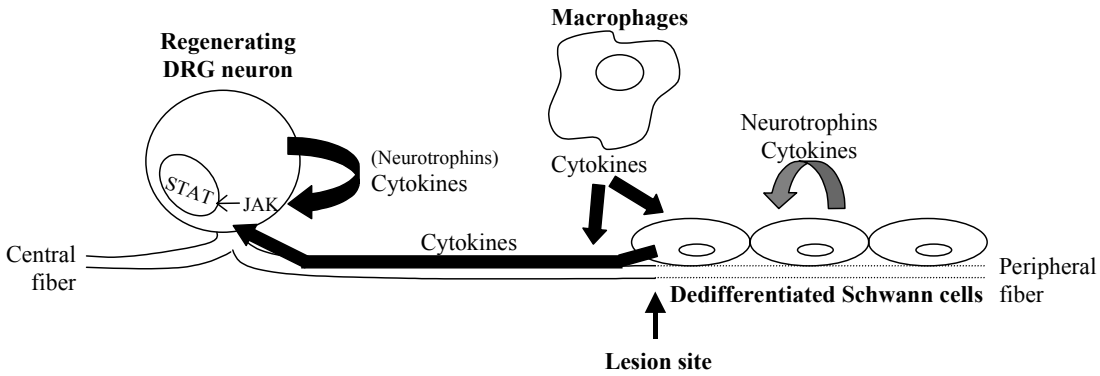

Figure 1: Neurotrophic support of DRG neurons and Schwann cells after sciatic nerve crush
Upon injury, dedifferentiated Schwann cells produce high amounts of neurotrophins and cytokines, the latter also produced by macrophages. Studies on signal transduction pathways revealed that the regenerating DRG neurons utilize neuroactive cytokines rather than neurotrophins for support of regenerative outgrowth [Liu and Snider, 2001; Markus et al, 2002]. The cytokines are retrogradely transported to the cell body [Curtis et al, 1994] and signal via JAK to the transcription factor STAT3, thus influencing gene expression in the regenerating neurons [Cafferty et al, 2001; Liu and Snider, 2001]. Neurotrophins seem to play only a minor role in outgrowth and survival of the injured neurons and are thought to be more important for the support of Schwann cells in the distal sciatic nerve [Mohiuddin et al, 1999].

Transcriptional mechanisms other than homeobox genes

The above-described differences in the signal transduction pathways utilized during regeneration and development are likely to result in different transcriptional regulation of genes. Naturally, certain developmentally expressed genes, like genes involved in cytoskeletal reorganization and stabilization, are regulated during regeneration, because these changes are necessary for the structural regrowth of axons of the DRG neurons. However, the transcriptional mechanisms utilized to regulate the expression of these genes should then be linked differently to signal transduction pathways during regeneration and development. Indeed, Udvadia et al (2001) showed in the zebrafish that a 1 kb fragment of the *B-50* promoter of the rat, directing *B-50* expression during development, was not capable of reactivating *B-50* during regeneration. This indicates that a different part of the *B-50* promoter is used during regenerative axon outgrowth as compared to development. Although this work has been done on zebrafish optic nerve, the same mechanisms may be true for the mammalian sciatic nerve, as CNS neurons in zebrafish are capable of regeneration [Udvadia et al, 2001].

As stated before, differences in signal transduction pathways are likely to result in differences in gene transcription between regeneration and development. There are not many reports on regeneration-associated transcription factors in DRG neurons (Table 1). The first transcription factor that has been shown to be upregulated in DRG neurons after nerve crush was *c-jun*. Interestingly, *c-fos* is not upregulated in DRG neurons [Leah et al, 1991; Plantinga et al, 1994; Soares et al, 2001]. c-Jun and c-Fos are known to be upstream of genes with activator protein-1 (AP-1) binding sites in their promoter, among which is *B-50* [de Groen et al, 1995]. This finding also marks *B-50* as an important target gene of c-Jun with respect to regeneration [Herdegen and Leah, 1998 and references therein]. The increase in *c-jun* is among the earliest reactions described and seems to coincide with the onset of the cell body response to injury. During development subsets of DRG neurons show only moderate expression of c-Jun, retained at constitutively low levels in adulthood [Herdegen and Leah, 1998, and references therein]. The upregulation of *c-jun* in virtually all injured cells, therefore, does not reflect a recapitulation of development.

Another transcription factor described in regeneration is activating transcription factor 3 (ATF3). ATF3 is closely related to c-jun, and activates transcription as a heterodimer with c-jun. *ATF3* mRNA is not expressed in embryonic or adult DRGs, but is induced after nerve injury, mostly in the same cells that upregulated c-jun. In general, *ATF3* expression is induced in many cell types in response to stress signals, indicating that its activation is not specific to nerve injury [Tsujino et al, 2000]. Finally, a recent report of Kabos et al (2002) on basic helix-loop-helix transcription factors, showed that *Sharp2* and *HES1*, negative regulators of developmental neurite outgrowth, are downregulated in DRG neurons, but positive regulators, *neurogenins*, are not induced (Table 1).

Conclusions

Taken together, in this thesis I have demonstrated that during regeneration of the crushed sciatic nerve, DRG neurons do not simply recapitulate the developmental expression patterns of homeobox genes that are functionally involved in the embryonic DRGs in neuronal differentiation, axonal outgrowth, pathfinding, and neuronal survival. The transcriptional regulation of these processes during regeneration is, therefore, fundamentally different from development. There is evidence that adult DRG neurons utilize neuroactive cytokines rather than neurotrophins for regenerative outgrowth and survival (Figure 1).

The preferential induction of target genes downstream of cytokine rather than neurotrophin signal transduction pathways underscores this notion. Transcription factors other than homeobox genes that are induced during regeneration also reflect a difference between regenerating and developing DRGs.

I would like to propose that in the adult DRGs, regeneration-specific mechanisms exist, inducing regeneration-specific gene expression in DRG neurons. These mechanisms may reflect adult gene regulation rather than developmentally controlled gene expression, and are partly imposed by the different context of adult versus embryonic DRG neurons. Differences in the neuronal environment implicate different signaling molecules and different signal transduction, leading to a different gene expression program in regenerating as opposed to developing DRG neurons. In future studies, further unraveling of the signal transduction pathways in regenerating DRG neurons should provide more insight into those mechanisms that are regeneration-specific. This may result in novel strategies to promote the regeneration process.

Next pages:

Table 1: Regeneration-associated alterations in gene expression in specific cell types after injury of the sciatic nerve.

Genes are classified according to their function and ordered chronologically according to their date of publication. Only the genes expressed in DRGs and sciatic nerve cells are shown; the spinal cord motor neurons are left aside. The types of injury are also not considered, assuming that similar gene expression changes occur after crush lesion, transection, ligation or other types of injury to the nerve fibers. Most of the changes shown here are based upon mRNA studies, some are based on immunocytochemical studies, or on phosphorylation (P) or activity (A) measurements. Finally, studies using microarrays or differential display were not included in this table unless the cell types in which gene expression alterations occurred were checked. For detailed information from gene expression profiling studies see Bosse et al, 2001; Costigan et al, 2002; and Xiao et al, 2002.

SN = DRG neurons
SC = Schwann cells
sat = satellite cells
mϕ = macrophages
en = endothelial cells
F = fibroblasts
# = Sciatic nerve, cell type not specified.

* = Dual response: upregulation in small SN, downregulation in large SN
** = Dual regulation: first up- then downregulated
*** = Dual regulation: first down- then upregulated

If conflicting data exist these are indicated in the references column

Genes	Up	Down	Cell types	References
Cytoskeletal (-associated) proteins				
Actin	x		SN	Nielsch and Keen, 1988
Beta-tubulin, type II, III	x		SN	Hoffman and Cleveland, 1988; Oblinger et al, 1989; Muma et al, 1990; Moskowitz et al, 1993 Jiang et al, 1994; Moskowitz and Oblinger, 1995; Hoffman and Luduena, 1996
Alpha-tubulin, type I	x		SN	Miller et al, 1989
Neurofilament -H, -M, and -L		x	SN	Hoffman and Cleveland, 1988; Oblinger et al, 1989; Muma et al, 1990; Jiang et al, 1994
Vimentin	x		SC	Neuberger and Cornbrooks, 1989
GFAP	x	x	SC	Neuberger and Cornbrooks, 1989; Quattrini et al, 1996, conflicting
C4 and S-100		x	SC	Neuberger and Cornbrooks, 1989
B-50/GAP-43	x		SN, SC	Van der Zee et al, 1989; Woolf et al, 1990; Plantinga et al, 1993a&b
Peripherin	x		SN	Wong and Oblinger, 1990
High molecular weight Tau		x,-	SN	Oblinger et al, 1991; Nothias et al, 1995, conflicting
Gelsolin	x		SC	Tanaka and Sobue, 1994
Beta-actin	x		SN	Lund and McQuarrie, 1996
KIF1A/1B/3A/3B/5		x	SN	Takemura et al, 1996
Microtubule-associated protein 1B (MAP1B)	x	P	SN, SC	Ma et al, 2000
MAP1B phosporylation mode I	x		SN, SC	Bush et al, 1996, Ramon-Cueto and Avila, 1999
MAP1B phosporylation mode II		x	SN, SC	Bush et al, 1996, Ramon-Cueto and Avila, 1999
Neurofilament -M, and -L	x		SC	Fabrizi et al, 1997
SCG10 and CAP23	x		SN	Mason et al, 2002
Small proline-rich repeat protein 1A and S100C	x		SN	Bonilla et al, 2002
Caveolin-1		x	SC	Mikol et al, 2002
SNAP-25A		x	SN	Costigan et al, 2002; Xiao et al, 2002

Table 1

Genes	Up	Down	Cell types	References
Extracellular matrix proteins and receptors				
Collagen, type I, III, IV	x		F	Siironen et al, 1992a&b; Nath et al, 1997
Laminin beta1	x		F	Siironen et al, 1992b
Beta 4 integrin	x		SC	Quattrini et al, 1996
Beta 1 integrin	x		F	Taskinen et al, 1995
Laminin beta2	x		SN, SC, sat	Le Beau et al, 1995
F-spondin	x		#	Burstyn-Cohen et al, 1998
Heparin-binding adhesive glycoprotein p200	x		SC	Chernousov et al, 1999
Fibronectin splice variants	x		SC	Vogelezang et al, 1999
Thrombospondin	x		SC	Hoffman and O'Shea, 1999
Beta-dystroglycan		x	SC	Masaki et al, 2000
Laminin-alpha2		x	SC	Masaki et al, 2000
Laminin-2 ($\alpha 2\beta 1\gamma 1$) and -8 (a4b1g1)	x		#	Wallquist et al, 2002
Cell adhesion molecules				
NCAM and L1	x		SC	Martini and Schachner, 1988; Tacke and Martini, 1990
J1/Tenascin	x		F, SC	Martini et al, 1990
E-cadherin***	x	x	SC	Hasegawa et al, 1996; Tada et al, 2001***
Ninjurin2	x		SC	Araki and Milbrandt, 2000
Close homologue of L1 (CHL1)	x		SN	Zhang et al, 2000
Attractants, repellents and receptors				
Netrin-1	x		SC	Madison et al, 2000
Neuropilin-1	x		SN	Gavazzi et al, 2000, conflicting with Pasterkamp et al, 1998

Genes	Up	Down	Cell types	References
Neurotrophins and receptors				
Nerve growth factor (NGF)	x		SC, sat, SN	Heumann et al, 1987; Meyer et al, 1992; Lee et al, 1998; Sebert and Shooter, 1993
High-affinity NGF receptor TrkA		x	SN	Verge et al, 1989; Verge et al, 1996
Brain-derived neurotrophic factor (BDNF)	x		SC, SN	Meyer et al, 1992; Funakoshi et al, 1993; Sebert and Shooter, 1993
Neurotrophin-3 (NT-3)		x	SC	Funakoshi et al, 1993; Cai et al, 1998
Neurotrophin-4 (NT-4)	x		SC	Funakoshi et al, 1993
High-affinity BDNF receptor TrkB (full length)	x		SN	Foster et al, 1994, immunoreactivity, conflicting with chapter 3
Low-affinity neurotrophin receptor p75	x		SC	Bolin and Shooter, 1993; Zhou et al, 1996
High-affinity BDNF receptor TrkB (truncated)		x	SC	Funakoshi et al, 1993
High affinity NT-3 receptor TrkC (truncated)		x	SC	Funakoshi et al, 1993
Low-affinity neurotrophin receptor p75		x	SN	Zhou et al, 1996
Other growth factors and receptors				
Epidermal growth factor receptor (EGFR)	x		F, SC	Toma et al, 1992
Glial cell line-derived neurotrophic factor (GDNF)	x		SC	Trupp et al, 1995; Naveilhan et al, 1997
Insulin-like growth factor-I (IGF-I)	x		SC, mϕ	Pu et al, 1995; Cheng et al, 1996
Insulin-like growth factor-II (IGF-II)	x		SC	Pu et al, 1995
IGF binding protein 5 (IGFBP5)	x		SC	Cheng et al, 1996
Neuregulin	x		SC	Carroll et al, 1997
Neuregulin receptors, ErbB2, ErbB3	x, P		SC	Carroll et al, 1997; Kwon et al, 1997
GDNF receptor (GDNFR) alpha	x		SC	Naveilhan et al, 1997
Ret	x		SN	Naveilhan et al, 1997
Reg2	x		SN	Livesey et al, 1997; Averill et al, 2002
Epidermal growth factor receptor (EGFR)	x		SN	Xian and Zhou, 1999
GDNF receptor (GFR) alpha-1 and -3	x		SN	Bennett et al, 2000; Höke et al, 2002
GDNF receptor (GFR) alpha-2	x	x	SN	Bennett et al, 2000; Höke et al, 2002, conflicting

Table 1

Genes	Up	Down	Cell types	References
Cytokines and receptors				
Tissue plasminogen activator (tPA)		x	SC	Neuberger and Cornbrooks, 1989
Ciliary neurotrophic factor (CNTF)		x	SC	Rabinovsky et al, 1992; Friedman et al, 1992; Smith et al, 1993
Leukemia inhibitory factor (LIF)	x		SC	Curtis et al, 1994; Dowsing et al, 1999
Transforming growth factor (TFG) -beta1	x		SC, mϕ	Rufer et al, 1994; La Fleur et al, 1996; Ryoke et al, 2000
Basic fibroblast growth factor (bFGF)	x		SN, SC, sat	Ji et al, 1995; Meisinger and Grothe, 1997
Interleukin-6 (IL-6)	x		SC, SN	Bolin et al, 1995
Tumor necrosis factor (TNF) alpha	x		SC, mϕ	La Fleur et al, 1996; Shamash et al, 2002
Monocyte chemoattractant protein-1 (MCP-1)	x		SC, en	Toews et al, 1998; Carroll and Frohnert, 1998; Taskinen and Roytta, 2000
Transforming growth factor (TGF)-alpha	x		sat	Xian and Zhou, 1999
Macrophage migration inhibitory factor (MIF)	x		SN, SC, F, en	Nishio et al, 1999; Taskinen and Roytta, 2000
LIF receptor subunit beta and glycoprotein 130	x		SC	Dowsing et al, 1999
Interleukin (IL) -1 beta	x		#	Ryoke et al, 2000
RANTES	x		en, F, mϕ	Taskinen and Roytta, 2000
IL-11 and Oncostatin	x		#	Ito et al, 2000
IL-11 receptor alpha		x	#	Ito et al, 2000
IL-18	x		mϕ	Menge et al, 2001
Fibroblast growth factor (FGF) receptor 3	x		SN	Grothe et al, 2001
Fibroblast growth factor (FGF) -5	x		SC	Scarlato et al, 2001
Tranforming growth factor (TFG) -beta2	x		SN	Stark et al, 2001
Osteopontin	x		SC	Jander et al, 2002
IL-1 alpha&beta	x		SC	Shamash et al, 2002
Platelet-derived growth factor (PDGF) -B chain**	x	x	SC	Oya et al, 2002

Genes	Up	Down	Cell types	References
Neurotransmitters, -peptides and receptors				
Cholecystokinin (CCK)		x	SN	Shebab and Atkinson, 1986
Somatostatin (SOM)		x	SN	Shebab and Atkinson, 1986
Vasoactive intestinal polypeptide (VIP)	x		SN	Shebab and Atkinson, 1986; Villar et al, 1989; Xu et al, 1990
Galanin	x		SN	Villar et al, 1989; Xu et al, 1990; Hu and McLachlan
Preprotachykinin (ppta) * / Substance P (SP)	x	x	SN	Villar et al, 1989; Henken et al, 1990; Noguchi et al, 1994*
Neuropeptide (NPY)	x		SN	Wakisaka et al, 1991
Neuronal nitric oxide synthase (nNOS)	x		SN	Fiallos-Estrada et al, 1993; Gonzalez-Hernandez and Rustioni, 1999a&b
Calcitonin gene-related peptide (CGRP)		x	SN	Hökfelt et al, 1994; Groves et al, 1996
Peripheral benzodiazepine receptor (PBR)	x		SC, mϕ	Lacor et al, 1996; Lacor et al 1999
Octadecaneuropeptide (ODN)	x		SC	Lacor et al, 1996
Islet amyloid polypeptide (IAPP)		x	SN	Mulder et al, 1997
Purine receptor P2X3		x	SN	Bradbury et al, 1998
Angiotensin receptor 1b and 2	x		SN, SC	Gallinat et al, 1998
Angiotensin receptor 1a	x		SC	Gallinat et al, 1998
Endothelial nitric oxide synthase (eNOS)	x		en	Gonzalez-Hernandez and Rustioni, 1999a&b
Inducible nitric oxide synthase (iNOS)	x		mϕ	Gonzalez-Hernandez and Rustioni, 1999a&b
Alpha2A adrenergic receptor	x		SN	Birder and Perl, 1997
Serotonin receptors		x	SC, SN	Yoder et al, 1997; Costigan et al, 2002
Purine receptor P2Y1	x		SN	Xiao et al, 2002
Nicotinic Acetylcholine receptorα7 subunit	x		SN	Xiao et al, 2002
$GABA_A$ Receptor α5 subunit	x		SN	Xiao et al, 2002
Peripheral benzodiazepine receptor	x		SN	Xiao et al, 2002
Bradykinin B2 receptor	x		SN	Lee et al, 2002

Table 1

Genes	Up	Down	Cell types	References
Transcription factors				
Jun	x		SN	Leah et al, 1991; Fiallos-Estrada et al, 1993; Jenkins et al, 1993; Soares et al, 2001
Suppressed cAMP-inducible POU (SCIP)	x		SC	Scherer et al, 1994; Gondré et al, 1998
Oct2	x		SN	Begbie et al, 1996
Brn3a		SN	Begbie et al, 1996	
Isl1		x	SN	Hol et al, 1999
ATF3	x		SN	Tsujino et al, 2000
Mash2	x		SC	Küry et al, 2001a
Hoxb5, Hoxd3, Hoxa6		x	SC	Küry et al, 2001a
STAT3	P		SC	Sheu et al, 2000
Sharp2, Hairy/enhancer of split (HES) 1		x	SN	Kabos et al, 2002
Hormones and receptors				
Nuclear thyroid hormone receptors	x		SC	Barakat-Walter et al, 1993; Glauser L and Barakat-Walter, 1997
3 Beta-hydoxysteroid dehydrogenase		x	SC	Robert et al, 2001; Schumacher et al, 2001
Type 2 & 3 deiodinase	x		F	Li et al, 2001a&b
Kinases, phosphatases, signal transduction				
Protein-tyrosine kinase pp60c-src and targets	x, P		SC, SN	Le Beau et al, 1991; Ignelzi et al, 1992
Mitogen activated protein kinase (MAP-K)	x		SC, SN	Svensson et al, 1995; Kim et al, 2002
cAMP phosphodiesterase	x, A		SC	Walikonis RS and Poduslo, 1998
Adenylyl cyclase		A	SC	Walikonis RS and Poduslo, 1998
Protein tyrosine phosphatase (PTP) alpha		x	SN	Haworth et al, 1998
Protein tyrosine phosphatase (PTP) sigma	x		SN	Haworth et al, 1998
Leukocyte common antigen-related protein tyrosine phosphatase receptor (LAR)		x	SN	Haworth et al, 1998; Xie et al, 2001
ERK1/2	P		SC	Sheu et al, 2000; Abe et al, 2001

Genes	Up	Down	Cell types	References
Channels/transmembrane proteins				
Potassium channels, MK1 and MK2		x	SC	Chiu et al, 1994
Connexin32		x	SC	Chandross et al, 1996; Nagaoka et al, 1999
Connexin43	x		F	Chandross et al, 1996; Nagaoka et al, 1999
Connexin46	x		SC	Chandross et al, 1996
Alpha2 and Beta2 subunits of Na+, K+-Atpase	x, A		SC	Kawai et al, 1997
Connexin26**	x	x	#	Nagaoka et al, 1999
L-calcium channel$\alpha 2\delta$-1	x		SN	Xiao et al, 2002
Sodium channel$\beta 2$	x		SN	Xiao et al, 2002
Calcium channelα-2 subunit	x		SN	Costigan et al, 2002
Proteases and protease regulators				
Glial-derived nexin	x		SC	Meier et al, 1989
Endopeptidase-24.11		x	SC	Kioussi et al, 1995
Matrix metalloproteinases (MMP) -2, -3 and -9	x		SC, mϕ	La Fleur et al, 1996; Ferguson and Muir, 2000
Tissue inhibitor of MMPs (TIMP-1)	x		SC, mϕ	La Fleur et al, 1996
Cellular metalloprotease disintegrin (rMDC15)	x		SC, SN	Bosse et al, 2000
Plasminogen activators	x		SN	Siconolfi and Seeds, 2001
Damage-induced neuronal endopeptidase (DINE)	x		SN	Kato et al, 2002
Myelin proteins				
P0		x	SC	LeBlanc and Poduslo, 1990; Mitchell et al, 1990; Gupta et al, 1993
Myelin basic proteins (MBPs)		x	SC	LeBlanc and Poduslo, 1990; Mitchell et al, 1990; Gupta et al, 1993
Myelin-associated glycoprotein (MAG)		x	SC	LeBlanc and Poduslo, 1990; Mitchell et al, 1990; Gupta et al, 1993
P2		x	SC	LeBlanc and Poduslo, 1990
Plasmolipin		x	SC	Gillen et al, 1996

Table 1

Genes	Up	Down	Cell types	References
Miscellaneous enzymes				
Aldose reductase		x, A	SC	Wong et al, 1992
2',3'-Cyclic nucleotide 3'-phosphodiesterase		x	SC	LeBlanc et al, 1992
NADPH-diaphorase	x, A		SN	Fiallos-Estrada et al, 1993
Beta-1,4-galactosyltransferase II and V	x		SC	Shen et al, 2002
GTP cyclohydrolase	x		SN	Costigan et al , 2002
Miscellaneous genes				
Apolipoprotein E	x		#	LeBlanc and Poduslo, 1990
Apolipoprotein D	x		F	Spreyer et al, 1990, Kim et al, 2001
Beta-amyloid precursor protein (APP)	x		SN	Scott et al, 1991
Hemopexin	x		SC, F, mϕ	Swerts et al, 1992; Madore et al, 1994; Camborieux et al, 1998
PMP22		x	SC	Kuhn et al, 1993
FKBP-12	x		SN	Lyons et al, 1995
Lactoseries oligosaccharides		x	SN	Groves et al, 1996
Monoclonal antibody 4C5 antigen	x		SC	Thomaidou et al, 1996
Clusterin and complement factor C4	x		#	Bonnard et al, 1997
Cyclin D1	x		SC	Atanasoski et al, 2001
Heat shock protein (Hsp) 27	x, P		SN	Kim et al, 2001; Benn et al, 2002
Immediate-early serum-responsive JE	x		SN	Costigan et al , 2002
Nerve growth factor-inducible protein (VGF)	x		SN	Costigan et al , 2002

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Abbreviations

BDNF	brain-derived neurotrophic factor
bp	basepairs
Brn	brain
cDNA	copy DNA
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
C_t	cycle threshold
DNA	deoxyribonucleic acid
dpo	post-operative day
DRG	dorsal root ganglion
DRGs	dorsal root ganglia
E*n*	embryonic day *n*
FGF	fibroblast growth factor
GAP-43	growth-associated protein-43
GDNF	glial cell line-derived neurotrophic factor
Gsc	goosecoid
HD	homeodomain
HE	hematoxylin eosin
IGF	insulin-like growth factor
IL	interleukin
Isl	islet
L	lumbar
LIF	leukemia inhibitory factor
LIM	Lin-11, Isl-1, Mec-3
mRNA	messenger RNA
NGF	nerve growth factor
NT	neurotrophin
P0	postnatal day 0
PCR	polymerase chain reaction
PNS	peripheral nervous system
POU	Pit-1, Oct1 and 2, Unc86
r	rhombomer
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SP	substance P
TGF	transforming growth factor
Trk	tyrosine kinase receptor

Nederlandse samenvatting

Het zenuwstelsel is opgebouwd uit van neuronen, die met elkaar communiceren via hun vezels. Tijdens de embryonale ontwikkeling treden processen op als migratie - waardoor neuronale stamcellen op de juiste plek terechtkomen - en specificatie en differentiatie - waardoor neuronen hun specifieke fenotype verkrijgen. De neuronen groeien uit en de zenuwvezels bereiken door middel van "pathfinding" (het vinden van de juiste route) hun doelwitorganen of doelwitneuronen. Om deze embryonale processen in goede banen te leiden, worden genexpressie-programma's in gang gezet door transcriptiefactoren, waaronder homeoboxgenen. Homeobox-genen coderen voor homeodomeineiwitten, die met behulp van hun DNA-bindingsdomein of homeodomein binden aan de promotoren van genen waarvan ze de expressie reguleren. Homeoboxgenen zijn betrokken bij veel processen in de embryonale ontwikkeling: "patterning" van het zenuwstelsel, specificatie en differentiatie van neuronen, uitgroei, pathfinding en overleving van neuronen. Het is reeds aangetoond dat homeoboxgenen ook in het volwassen zenuwstelsel tot expressie komen, maar de functies van homeoboxgenen in volwassen neuronen zijn nog niet bekend. Gedacht wordt dat ze een rol spelen bij het instandhouden van neuronen, dus bij neuronale overleving. Ook is het mogelijk dat ontwikkelingsprocessen in het volwassen zenuwstelsel zouden kunnen optreden. In dit proefschrift is onderzocht of dat het geval is voor het herstel - regeneratie - van zenuwcellen van het perifere zenuwstelsel na schade.

Het perifere zenuwstelsel wordt onder andere gevormd door dorsale streng ganglia, of "dorsal root ganglia (DRG's). Perifere neuronen in de DRG's voorzien het centraal zenuwstelsel van sensibele informatie van de huid, darmen en spieren. Ze verzorgen onder andere de waarneming van pijnprikkels, temperatuur en stand van het lichaam. Ze vinden hun oorsprong in de neurale lijst cellen die tijdens de embryonale ontwikkeling migreren naar verschillende plaatsen in het lichaam om daar specifieke neuronale structuren te vormen, onder andere DRG's. De neuronen in de DRG's differentiëren vervolgens tot verschillende subpopulaties die uitgroeien naar hun perifere doelwitorganen.

Een eigenschap van perifere zenuwen die deze onderscheidt van het centraal zenuwstelsel, is de mogelijkheid tot herstel na schade aan de zenuw in het volwassen organisme. Dit regeneratieproces houdt in dat, na zogenaamde Walleriaanse degeneratie, de zenuwcellen opnieuw gaan uitgroeien. Daardoor worden de doelwitorganen opnieuw geïnnerveerd en wordt de zenuwfunctie hersteld. In dit proefschrift is gebruik gemaakt van het "sciatic nerve crush" model. De nervus ischiadicus in de achterpoot van volwassen ratten of muizen wordt samengeknepen zodat de vezels distaal van de beschadiging degenereren en het regeneratieproces in gang wordt gezet. Dit proces gaat gepaard met veranderde genexpressie in DRG-neuronen. Bekend is dat genen die van belang zijn bij de embryonale neuronale uitgroei tijdens regeneratie opnieuw tot expressie worden gebracht. Voorbeelden hiervan zijn tubuline, actine en het groei-geassocieerde eiwit B-50. Genen die betrokken zijn bij de functie van de neuronen worden in hun expressie geremd.

Deze waarnemingen hebben geleid tot de hypothese dat de moleculaire mechanismen van regeneratie een herhaling zouden zijn van ontwikkelingsprocessen. In Schwann-cellen, de ondersteunende cellen van het perifere zenuwstelsel, is daadwerkelijk aangetoond dat ze een embryonaal fenotype aannemen en genen tot expressie brengen die tijdens de embryonale ontwikkeling actief geweest zijn. Hiertoe behoren onder andere de homeoboxgenen *Oct6* en *Pax3*. In neuronen echter, worden ook veranderingen in genexpressie aangetroffen die de ontwikkelingshypothese tegenspreken.

Het doel van de studies in dit proefschrift was dan ook om de hypothese dat regeneratie van DRG-neuronen teruggrijpt op mechanismen die tijdens de embryonale ontwikkeling een rol spelen te onderzoeken door te kijken naar de expressie van homeoboxgenen. Aangezien deze transcriptiefactoren nauw betrokken zijn bij van processen als uitgroei, pathfinding en neuronale overleving tijdens de embryonale ontwikkeling ligt het voor de hand dat, als er een herhaling optreedt van ontwikkelingsprocessen, de expressie van homeoboxgenen in regenererende neuronen vergelijkbaar is met die in embryonale DRG's.

Om een beeld te krijgen van het repertoire aan homeoboxgenen in het DRG van de volwassen rat is in hoofdstuk 2 een inventarisatie gemaakt. Met een op PCR gebaseerde methode zijn twee en twintig homeoboxgenen geïdentificeerd, waarvan er zes met zekerheid konden worden gelokaliseerd in DRG-neuronen met behulp van *in-situ*-hybridisatie. Het expressieniveau van veel van deze homeoboxgenen bleek dusdanig laag te zijn dat deze genen niet met de conventionele histologische technieken waren aan te kleuren. Hierdoor is het waarschijnlijk dat de huidige kennis over de expressie van transcriptiefactoren in het DRG verre van compleet is. De diversiteit aan homeoboxgenen die tot expressie komen in het volwassen DRG suggereert dat de regulatie van genexpressie tijdens de ontwikkeling ook in volwassen DRG's blijft bestaan.

Om de regulatie van een select aantal homeoboxgenen tijdens regeneratie te onderzoeken, is de overstap gemaakt van de rat naar de muis als proefdier. De muis heeft als voordeel dat de expressie van homeoboxgenen kan worden gerelateerd aan functionele aspecten, die waarneembaar zijn in mutante muizen. Deze mutanten zijn meestal verkregen door "knock-out" studies (studies in muizen die een (homeobox) gen missen). Omdat de C57BL/6J-muizenstam vaak voor knock-out studies wordt gebruikt, is deze muis gekozen om de expressie van homeoboxgenen tijdens regeneratie in te onderzoeken. Omdat er in de literatuur twijfels bestonden over de regeneratieve capaciteit van deze stam is eerst een karakterisering gedaan van het regeneratie-model in C57BL/6J-muizen (hoofdstuk 3). Het verloop van het herstel van sensibele en motorische functies kwam overeen met dat in de rat. Er was geen verlies van neuronale subpopulaties en bekende veranderingen in genexpressie konden worden gereproduceerd. Hieruit concludeerden we dat de C57BL/6J-stam gebruikt kon worden om veranderingen in genexpressie te onderzoeken in verband met regeneratie.

Hoofdstuk 4 beschrijft de kwantitatieve analyse van een aantal homeoboxgenen, *DRG11*, *Gsc*, *Isl1*, *Lmx1b*, *Otp* en *Pax3*, in de C57BL/6J-muizen, op drie tijdstippen na de "crush"-beschadiging. We hebben gevonden dat de expressieniveaus van *Isl1* tijdelijk dalen. Echter, *Isl1* komt in het embryonale DRG tot expressie in meer neuronen dan in het volwassen DRG. Als regeneratie een recapitulatie zou zijn van ontwikkeling zou een stijging van de expressie te verwachten zijn. De niveaus van *DRG11*, *Lmx1b* en *Pax3* bleven constant. Van *DRG11* en *Pax3* is bekend dat ze tijdens de embryonale ontwikkeling een rol spelen bij de doelwitinnervatie en differentiatie van DRG-neuronen. Net als *Isl1* komt *DRG11* in het embryo in meer neuronen tot expressie dan in het volwassen DRG. Expressie van *Pax3* is ook eerder aangetoond in embryonale DRG-neuronen, maar in de volwassen DRGs bleek *Pax3* extreem laag in niveau te zijn. De constante niveaus van *DRG11* en *Pax3* tonen aan dat de volwassen expressiepatronen worden gehandhaafd. Dit wordt onderschreven door *Lmx1b*, een homeoboxgen dat niet tot expressie komt in het embryonale DRG, maar wel in volwassen DRG-neuronen (hoofdstuk 2). Hoewel de hypothese een daling in expressie voorspelt, veranderde ook de expressie van *Lmx1b* niet na crush. We concludeerden hieruit dat de expressie van homeoboxgenen in regenererende neuronen verschilt van die in embryonale DRG-neuronen.

De homeoboxgenen *Gsc* en *Otp* bleken sterk te variëren in expressieniveau, zowel binnen als tussen experimentele groepen. Vermoedelijk bestaat er een klein aantal neuronen dat deze genen tot expressie brengt en is dit aantal variabel verdeeld over verschillende DRG's. Door deze variatie kon geen conclusie worden getrokken over een eventuele rol bij regeneratie. De expressie van zowel *Gsc* als *Otp* in het DRG is nog niet eerder beschreven. Van *Gsc* is bekend dat deze van belang is voor de ontwikkeling van mesectodermale derivaten van de neurale lijst. Omdat DRG's ook afkomstig zijn van neurale lijst cellen, is in hoofdstuk 5 onderzocht of *Gsc* betrokken is bij de ontwikkeling van het DRG. Na aangetoond te hebben dat *Gsc* ook in de ontwikkelende DRG's tot expressie komt, laten we zien dat in neonatale *Gsc*-knock-out-muizen de belangrijkste neuronale subpopulaties aanwezig zijn in het DRG. Daarnaast heeft de *Gsc*-mutatie geen invloed op de expressie van andere homeoboxgenen in DRG-neuronen. We concludeerden uit hoofdstuk 5 dat de functie van *Gsc* verschillend is in de ontwikkeling van verschillende neurale lijst derivaten.

Uit de resultaten die in dit proefschrift beschreven staan, blijkt dat de veranderingen in de expressie van homeoboxgenen in het DRG na zenuwschade niet corresponderen met de expressiepatronen van deze genen tijdens de embryonale ontwikkeling. Dit betekent dat regeneratie van DRG-neuronen, in tegenstelling tot Schwann-cellen, niet simpelweg een proces is van de- en redifferentiatie. De transcriptionele mechanismen die ten grondslag liggen aan de processen differentiatie, uitgroei, pathfinding en overleving, in het embryo aangestuurd door *Brn3a*, *DRG11*, *Pax3*, en mogelijkerwijs *Isl1*, zijn dus verschillend in regenererende DRG-neuronen. Naast homeoboxgenen wijzen ook andere waarnemingen in de richting van regeneratie-specifieke genexpressieprogramma's. Door in te grijpen in de signaaltransductie is aangetoond dat, in tegenstelling tot embryonale uitgroei, regeneratieve uitgroei van DRG-neuronen niet afhankelijk blijkt te zijn van neurotrofines, maar van neuroactieve cytokines. Ook op het niveau van genexpressie lijken neurotrofines geen direct effect te hebben op DRG-neuronen. Genen die door, bijvoorbeeld, NGF worden gestimuleerd, worden toch verminderd tot expressie gebracht in regenererende neuronen. Daarentegen worden genen waarvan bekend is dat ze door de cytokines worden gestimuleerd wel verhoogd tot expressie gebracht na zenuwschade. Een belangrijke transcriptiefactor in de signaaltransductieroute van neuroactieve cytokines, STAT3, wordt dan ook in gefosforyleerde (actieve) vorm aangetroffen in regenererende neuronen. De activatie van andere transcriptiefactoren, zoals c-Jun en ATF3, die wel tijdens regeneratie maar niet of nauwelijks tijdens de embryonale ontwikkeling tot expressie worden gebracht, onderschrijft de conclusie dat de transcriptie in regenererende DRG-neuronen verschilt van die in embryonale DRG-neuronen.

Samengevat, heb ik in dit proefschrift aangetoond dat DRG-neuronen tijdens regeneratie van de nervus ischiadicus niet simpelweg de embryonale expressie van homeoboxgenen herhalen. Daaruit volgt dat de transcriptionele regulatie van processen als differentiatie, uitgroei, pathfinding en overleving verschillend is in regenererende ten opzichte van embryonale DRG-neuronen. In het volwassen DRG bestaan regeneratie-specifieke mechanismen, die regeneratie-specifieke genregulatie tot gevolg hebben. Deze mechanismen worden gedeeltelijk veroorzaakt door verschillen in de omgeving van de neuronen, die leiden tot verschillen in signaaltransductie, transcriptie- en genexpressieprogramma's. In de toekomst moet verdere ontrafeling van de signaaltransductieroutes in regenererende DRG-neuronen meer inzicht geven in de regeneratie-specifieke mechanismen. Dit zou kunnen leiden tot nieuwe strategieën om het regeneratieproces te bevorderen.

Dankwoord

Thanx!

Het meest populaire “hoofdstuk” van mijn proefschrift, begin ik met iemand zonder wie ik nooit op het RMI terecht was gekomen. Loes, ik had je al gesproken tijdens de neuroscience cursus en had toen stiekem al verzonnen dat ik “bij haar wel AIO wilde worden”. Toen ik op zoek ging naar een baan viel mijn oog op een advertentie in het U-blad. Het project zag er leuk uit, maar jouw naam onder de advertentie gaf de doorslag. Niets te veel verwacht, je was de ideale baas. Altijd vrolijk, druk bezet en toch altijd tijd, want je was net als ik vaak vroeg. Je was m’n maatje, je vertrouwde me en steunde me als anderen het niet eens waren met m’n proeven: gewoon doen! Vooral in het eerste jaar zijn veel resultaten daaruit voortgekomen. Je kunt dit niet meer lezen en toch denk ik dat het “aankomt”. Het komt aan in de harten van alle mensen die van je hielden (houden) en die je waardeerden. Een emotioneel begin van mijn dankwoord, want het was een moeilijke tijd. Graag wil ik de mensen bedanken die mij gesteund hebben in deze periode. Rea, Jacqueline, Pierre en Willem Hendrik, bedankt dat ik bij de mensen hoorde, die als eerste ingelicht werden als er een nieuwe ontwikkeling was. Jullie zagen in dat ik me, al was het in maar 2 jaar, aan Loes gehecht had. Annie, ik had je pas twee keer ontmoet, bedankt voor het aanhoren van mijn “noodkreet” door de telefoon en voor het “verwerkingsdineetje”. Het contact over en weer via kaartjes en mails heb ik erg gewaardeerd. Jildau, bedankt voor de “loopjes”: even weg, even praten, uithuilen en dan weer verder, je was een enorme steun. Daniëlle, bedankt voor “het” telefoontje en medeleven. Marco, toen pas net begonnen, bedankt voor begrip en tactvolle overname van de praktische begeleiding; Peter, bedankt dat je deze oplossing bedacht hebt. Willem Hendrik, voor het “bemoedigend door elkaar schudden” vlak voor de plechtigheid en voor de mooie toespraak. Marjolein, ook jij was steun en toeverlaat, al was het over de mail. Gerard Jansen, als ik het echt niet meer uithield, kon ik een sprintje trekken naar jouw kamer. Het hele RMI, bedankt voor de verslagenheid en saamhorigheid. Bedankt!

Zo, na deze zware start over op een wat vrolijker vervolg. We hebben leuke dingen gedaan met z’n allen. Het DNA-culinaire Kerstdiner onder leiding van topkok Matthijs, bij Peter thuis. De labdagen, altijd goed voor een dosis cultuur gemixt met vrolijkheid, sportiviteit en lekker eten. Als het laat werd, Ria, mocht ik altijd met jou meerijden. Netjes afgezet voor het huis, geweldig bedankt. Samen met Tom, Jan en Wout in de Sinterklaascommissie veel plezier gehad. We hebben er met z’n viertjes een leuke avond van gemaakt en hebben elkaar een stuk beter leren kennen! De B-50 uitjes met de groep Pierre/Loes: van fietsen, kanoën, squashen, tot spelletjes, koken, muziek. Zo kom je er nog eens achter dat Jacqueline niet van spinnen houdt (aaah!), Henk heel koelbloedig spinnetjes verwijdert, Rea en Els leuke wandelpartners zijn. Henk, Govert en studenten onvermoeibaar zijn in voetballen (tot 2 uur ‘s nachts, bedankt!), Govert goed kan koken, Evelien fervent squasher is en Pierre kampioen pingpongen met effect! Jongens (en meisjes), het waren twee fantastische weekenden. Om nog even bij deze groep te blijven, Marina en Marije, labgenootjes op ons eigen stukje lab, altijd gezellig en behulpzaam. Leo, met jou erbij was de lunch altijd weer vrolijk. Rea, Jacqueline, crypto-maatjes, elke donderdag weer raak, koppie thee erbij en flink hardop denken. Pierre en Geert, bedankt voor het vertrouwen: dat ik als AIO-tje tot 3 keer toe een college mocht geven op de Neuroscience cursus vond ik een hele eer.

Nog een groep waar ik bij hoorde: de groep van Peter Burbach. Peter, Marten, Joke, Ceriel, Pilar, Arno, Marjan, Hikke, Anita, Marco, Simone, Patrick, Lars, Josine, Janine (eventjes), Cornelle, Agniezka, bedankt voor wetenschap en collegialiteit. Joke, toen nog analist, bedankt voor je enorme hulp bij het opstarten. Bij jou kon ik altijd aankloppen als ik ergens niet uit kwam. Ceriel, handig hoor, zo iemand die het een en ander aan reviews zo voor het grijpen heeft en zo veel informatie kan verschaffen over de te gebruiken primers en probes. Bedankt! Marjan, nog zo’n

reuzehulp, bedankt voor de tips voor het layouten en voor peptalk en enthousiasme. Marten (to be honest), ik was het niet altijd met je eens, maar toch bedankt voor de kritische kijk op de proeven. Zonder jouw hulp had ik nooit de goosecoid knock-out binnen kunnen halen, en ik heb veel van je geleerd. De etentjes bij jou waren geweldig. Marco, we konden het van het begin af aan goed met elkaar vinden, ik vond het fijn dat jij mijn co-promotor werd. Ideale begeleider wat betreft het schrijfwerk, je wist mijn gedachten in goede banen te leiden en zo over de zaken te discussiëren dat het gemakkelijker werd om ze op te schrijven. En ook al zo'n lekker etentje georganiseerd, hoewel Inês het meeste kookwerk had verricht (toch?). Josine, dat jij net als ik op de pathologie had stage gelopen, was erg leuk. Konden we lekker samen overleggen als we een protocol wilden aanpassen. De opmerkingen "op de patho…" waren niet van de lucht. Simone, begonnen als student bij mij, je hebt goed werk verricht, was enthousiast en voortvarend, ik vond het leuk om je als collega-AIO te mogen zien beginnen bij Marten. Johan, student nummer twee, je scriptie was een zware bevalling, had ik nooit zelf tijd voor gehad, bedankt. Jammergenoeg viel je project een beetje in het water, maar fijn dat je het bij Marco hebt kunnen afwerken.

Peter, als promotor zeer betrokken bij het verloop van mijn project ben je een geweldige steun geweest, vooral in tijden dat het minder ging. Het boekje zou er toch komen, je had gelijk. Hoe snel jij je door mijn laatste, zeer krap geplande schrijfperikelen heen werkte, alle lof en dank. En dan toch altijd een positieve draai eraan geven zelfs al werd een discussie van een bepaald hoofdstuk omgetoverd tot legpuzzel! Willem Hendrik (zonder streepje), promotor nummer twee, vooral in het begin bespraken we af en toe de loop van het project. We zaten aardig op één lijn, meestal was ik binnen 10 minuten weer weg. Je gevleugelde "Je mag niet zenuwachtig zijn!"…bij jou waren de zenuwen na een paar seconden altijd weer weg. En ik heb nou eenmaal trilhandjes. Dat je als rector magnificus toch nog af en toe tijd voor me had, waardeer ik zeer.

Een AIO komt er niet zonder praktische hulp. Zeker niet als je als moleculair bioloog af en toe tussen de dieren wordt gezet. Jan Brakkee, jouw expertise in het opereerwerk was onmisbaar. Ik ben er niet toe gekomen het zelf te leren. Samen met jou de muizencrush opzetten was geweldig, en het record staat nog, hè? Voor het klusje dat niemand leuk vindt, de beestjes moesten toch dood, kon ik altijd aankloppen bij Simone Duis, Henk, Leo of Jan. Heel erg bedankt. Frank Hamers, ik vond het ontzettend leuk om met jouw CatWalk opstelling de functionele metingen te doen. Bedankt voor je hulp, interesse, goede coaching bij het schrijven van hoofdstuk 3. Dorien, toen je mij met m'n trilhandjes de von Frey filamenten zag hanteren, besloot je om die metingen toch maar voor mij te doen, al was het na een lange dag in de kliniek. Geweldig bedankt. DNA-lab orakels Arno, Joke, Robbie, Sander en Tom, bedankt voor hulp en raad bij knippen, plakken, PCR-en, sequencen enzovoort. Maar ook zonder secretariaat is een AIO hulpeloos. Marijke, altijd geduldig als er iets niet klopte bij de proefdierbestellingen. Dick en Sjaak, de bestellingen, declaraties, faxjes, ze liepen allemaal goed met jullie hulp. Ria, bedankt voor alle hulp bij de formulierenboel en brieven.

Als je de middelen niet op eigen lab aanwezig hebt, dan ga je naar "de buren". De kwantitatieve PCR experimenten zijn gedaan op het lab van Jan Bogerd. Jan, bedankt voor alle hulp, adviezen, protocollen en voor het vertrouwen wat betreft sleutels en toegangspas. De warme ontvangst, snelle antwoorden op mails (inclusief het "even" selecteren van primers), het snelle lezen van hoofdstuk 4 en de nodige peptalk zal ik niet snel vergeten. Op datzelfde lab, Joke Granneman en Hans van Aken, bedankt voor praktische hulp en gezelligheid.

Kamergenoten bedanken is leuk in dit geval: het was een echte doorreiskamer. Jacqueline en Rea, heel gezellig, vooral voor het nodige cryptoplezier, wat vrolijk voortgezet werd toen jullie m'n

buren werden. Lars, Marjoleen, Janine, Cornelle en Koen, de ene voor langere tijd dan de andere. Martien, als postdoc op een AIO kamer zal je veel te verduren hebben gehad, bedankt voor geduld, interesse en adviezen. En natuurlijk Patrick/Patrique/Patje altijd vrolijk, gezellig en sociaal. Jij liep tenminste niet bij me weg….. Gitte, altijd in voor een babbel, crypto of snoepjes, je kwam er als laatste bij, maar was sneller ingeburgerd dan wie dan ook.

Joke, het is altijd prettig om met jou samen te werken, of het nu is op het lab, bij de bedrijfsvoering of in de milieucommissie. Ik vind het leuk dat je mijn paranimf wilde zijn. Heidi, paranimfje nummer twee, collega-AIO, altijd in voor een babbel en saampjes hebben we toch maar mooi die AIO avonden nieuw leven in geblazen. Jeroen, Patrick, Corine, Ineke, Jacqueline, Gert-Jan, Leontine en Daniel zonder jullie enthousiasme waren de AIO avonden ook niks geworden. Met het spel kolonisten erbij werden ze niet alleen nuttig maar ook errug leuk! Om bij de ontspanning te blijven, ook daar werd ruimschoots in voorzien. Rea, Jacqueline, Annemarie, Inge bedankt voor de theezakjes; Marjolein, ik ben nog steeds niet door je zoethoutmanie heen en Jildau, jouw smaak was afhankelijk van mijn voorkeuren (“Wat wil je, kaneel?”), geweldig! Ceriel, dat je mij aan het volleyballen kreeg, mag een wondertje heten. Samen met Diane en Jeroen was je een geweldige coach voor een meisje dat meedeed voor de gezelligheid maar er eigenlijk geen bal van kon!

Mensen waarmee je kunt lachen, huilen, geheimpjes delen en afspraken maken heten in de volksmond vrienden. Jildau, meid, wat hebben wij veel meegemaakt samen. Syndey congres en vakantie, samen koken, uitjes plannen, musea afgewisseld met natuur, op de een of andere manier wisten wij onze interesses goed te combineren. Dat je naar Amsterdam vertrok met de groep vond ik doodjammer, maar daar hebben we e-mail voor, openbaar vervoer en agenda’s om uitjes te plannen. Daniëlle, in het begin vond ik je maar een rare, maar je bent een lieve meid. Jeroen, je bent een toffe kerel en bedankt dat je Daniëlle gelukkig hebt gemaakt! Gerard, ik denk niet dat veel mensen nog zo’n goed contact hebben met hun vroegere stagebegeleider. Je bent meer dan dat, want ook wij hebben lief en leed gedeeld. Nu in Canada, maar daar heb je e-mail voor. Marjolein, ook het contact met jouw is e-mail-gebonden. Je lappen tekst zijn echt geweldig, je medeleven en steun in moeilijke tijden enorm. Bedankt.

Ten slotte komen gewoontegetrouw de belangrijkste mensen aan bod: familieleden. Weinig AIO’s wonen nog thuis, ik wel, dat zegt genoeg. Mamma, mijn beste vriendin, bij jou kan ik altijd terecht met problemen. Samen winkelen, samen koken, samen lachen en huilen, dingen die heel erg belangrijk zijn. Jij had biologie kunnen studeren, zo goed begreep je mijn onhandig uitgelegde labwerkzaamheden. Pappa, zoals een goed vader betaamt, snapte jij er minder van dan mamma. Maar van alle computerperikelen, brieven en formulieren des te meer. En als je meisje laat was en de bus miste.....zelfs in pyjama stapte jij nog achter het stuur. Hans, tweelingbroer, een band die niet met woorden te beschrijven valt. Trots op z’n zussie en ikke op jou. Oma, u zult er niet zijn op de promotie, maar u heeft dat van te voren ruimschoots gecompenseerd. Lieve schat, rust zacht en in mijn hart zult u altijd overal bij zijn, samen met opa.

Dat dit dankwoord drie paginas in beslag neemt zegt genoeg. Voor velen is de studententijd, voor mij is de AIO-tijd de mooiste van m’n leven geweest! Daarom voor iedereen op het RMI een welgemeend **dankjewel!**

CURRICULUM VITAE

Christina Francisca Vogelaar werd geboren op 13 november 1975 te Utrecht. Ze behaalde het VWO diploma in 1994 aan de F.A. Minkema Scholengemeenschap te Woerden. In datzelfde jaar begon ze met de studie Medische Biologie aan de Universiteit Utrecht. Na twee stages aan de vakgroep Pathologie, de eerste onder begeleiding van S.A. Scheltinga en Dr. M.G.J. Tilanus, de tweede onder begeleiding van neuropatholoog G.H. Jansen, behaalde ze in 1998 haar doctoraalexamen. In datzelfde jaar begon ze als assistent in opleiding bij de vakgroep Farmacologie en Anatomie van het Rudolf Magnus Instituut voor Neurowetenschappen onder begeleiding van Dr. L.H. Schrama, Prof. Dr. J.P.H. Burbach en Prof. Dr. W.H. Gispen. Na het overlijden van Dr. Schrama werd de praktische begeleiding overgenomen door Dr. M.F.M. Hoekman. De resultaten van het onderzoek, dat uitgaat van de vraag of regeneratie een recapitulatie van ontwikkeling is, zijn beschreven in dit proefschrift.

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