SENSORY NEURONS: STEM CELLS AND DEVELOPMENT

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Stockholm 2006
ABSTRACT

The sensory nervous system is the only means we have of communicating with the surrounding world. The neurons responsible for the sensation of pain, touch, the ability to know the position of our limbs and part of maintenance of body posture are located in the dorsal root ganglia (DRG). Stem cell biology has, during the recent years greatly enhanced our understanding of developmental processes. The aim of this thesis was to isolate and characterize stem cells from the sensory nervous system and to study the development of functional neuronal subtypes.

In the work presented I show the identification of a neural crest stem cell (NCSC) that is located in the boundary cap (BC). The BC is a transient structure present during embryogenesis lining the boundary between the peripheral and central nervous system at the exit/entry zone of sensory and motor efferents. This multipotent stem cell is unique as compared to previously described NCSCs, in its ability to form sensory neurons in vitro. The sensory neurons are functionally active as assayed by calcium imaging using temperature stimuli and sensory specific transient receptor potential (TRP)-channel ligands. I further show that the boundary cap neural crest stem cell (bNCSC) can give rise to Schwann cells that myelinate regenerating axons in vivo, suggesting a possibility for the use of these stem cells for regenerative therapy. The bNCSC express the well described stem cell marker, stage specific antigen 1 (SSEA-1) as well as proteins involved in the production of gamma amino butyric acid (GABA). Furthermore, GABA drastically reduces the proliferation of bNCSC, in a pathway independent of intracellular signalling. Antagonizing endogenous production using GABA$_\alpha$ receptor antagonist bicuculline increases the same. This suggests GABA as a signal to regulate proliferation in the BC stem cell niche and thus providing the basis for a possible increase of production in response to an injury. In the last part of the thesis I describe and define the developmental emergence of different subtypes of developing sensory neurons based on functional responses to capsaicin, menthol, and cinnamaldehyde, agonists to TRPV1, TRPM8 and TRPA1 respectively.

Published and printed by Reproprint AB
© Jens Hjerling-Leffler, 2006
ISBN 91-7140-667-0
Till Mamma och Malin
LIST OF PUBLICATIONS


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<th>Description</th>
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<tr>
<td>BC</td>
<td>Boundary cap</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>bNCSC</td>
<td>Boundary cap neural crest cell</td>
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<tr>
<td>CEE</td>
<td>Chick embryo extract</td>
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<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>DRG</td>
<td>Dorsa root ganglia</td>
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<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<td>FBn</td>
<td>Fibronectin</td>
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<td>GABA</td>
<td>Gamma amino butyric acid</td>
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<td>GDNF</td>
<td>Glial cell line derived neurotrophic factor</td>
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<tr>
<td>L</td>
<td>Lumbar</td>
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<td>Maob</td>
<td>Mono amino oxidase B</td>
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<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
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<tr>
<td>NC</td>
<td>Neural crest</td>
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<td>NCC</td>
<td>Neural crest cell</td>
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<td>NCSC</td>
<td>Neural crest stem cell</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NT3</td>
<td>Neurotrophin 3</td>
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<td>NT4/5</td>
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<td>OB</td>
<td>Olfactory Bulb</td>
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<td>P</td>
<td>Postnatal day</td>
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<tr>
<td>PEDF</td>
<td>Pigment epithelial-derived factor</td>
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<td>RA</td>
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<tr>
<td>SBZ</td>
<td>Sub granular zone</td>
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<tr>
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<td>Schwann cell</td>
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<tr>
<td>SDF1</td>
<td>Stromal cell-derived factor 1</td>
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<tr>
<td>SSEA-1</td>
<td>Stage specific embryonic antigen 1</td>
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<tr>
<td>SVZ</td>
<td>Sub ventricular zone</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Trk</td>
<td>Tropomyosin receptor kinase</td>
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<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1 OBJECTIVES

To:
- Study the stem cell properties of the boundary cap.
- Explore the potential for cell-based therapies for nervous system repair
- Study how the boundary cap stem cell niche is controlled
- Characterize the development of functional sensory neuron subtypes

2 INTRODUCTION

In this thesis I have bridged two fields of biology; sensory biology and developmental biology. Sensory biology is like the word implies the study of our senses. Everything we are is experienced through the windows of our five senses. Sight, smell, sound, taste and touch are the means we have to relate to our physical surroundings and together they create our Umwelt, a term coined by Estonian zoologist Jakob von Uexküll (Uexküll, 1909). Umwelt is the world we perceive which in addition to the total input from sensory organs, also includes how this information is processed. A lot of effort has been put into understanding the senses on psychological as well as mechanistic levels. For example, we have vast knowledge on how the photo energy from our surrounding is deciphered in the eye with its photoreceptors and begin to understand more about how this information is processed in the central nervous system (Wald, 1935). In regards to smell, the recent discoveries leading to the identification of the protein family responsible for odor reception could hardly have escaped anyone (Buck and Axel, 1991). Both of these seminal discoveries were rewarded with the Nobel Prize in 1967 and 2004 respectively. An increasing number of stimulants of the olfaction system are being described and characterized (Stensmyr et al., 2003) however many pieces in the puzzle remain missing, especially with regards to central processing and how the system assembles during development. The latter statement holds true also for the sensation of touch, the sense discussed in this thesis. The receptive organs are known and described anatomically but, with the exception of detection of thermal energies (heat/cold), little is known about what mediates the detection of haptic stimuli on a subcellular level. The cells responsible populate the dorsal root ganglia (also known as spinal ganglia) and send their afferent projections out into peripheral target organs (e.g. muscles, skin or intestines) and efferents into the spinal cord for central processing.

Developmental biology is the study of how organisms grow and mature. The first formulated question in the records is from the 5th century, when the Greek philosopher Aristotle formulated the question on how the different parts of an embryo were formed. Was the body plan already laid out and then only grew, or was it through a process similar to the “knitting of a net” or epigenesis? This
question was still being debated throughout the 18th century and it was not until the emergence of the cell theory in early 19th century that the preformation theory was discarded. The discovery of cells however raised a multitude of questions, with the central theme: how a single cell can become an adult functional animal with millions of cells? Developmental biology, in the middle of last century, exploded with the finding of the transcription factors, molecules involved in every aspect of development. Knowledge in developmental biology can help us to understand diseases or syndromes caused by developmental defects. This study is focused on the later stages of embryology, during which the peripheral nervous system is set up but I provide give a brief introduction on how the neural crest gives rise to the sensory system including the boundary cap stem cells.

2.1 THE NEURAL CREST

2.1.1 Discovery and evolutionary origin
The neural crest is a transient cell population that arises from the border between ectoderm and endoderm after gastrulation, and it was first described in chick embryos (His, 1868). It is unique in its abilities to form a variety of cell types and to migrate throughout the entire embryo. Several studies using different tracing techniques have been performed to elucidate what structures these cells give rise to, and the list has grown long, including bone and cartilage in the cranium, pigment cells of the skin, chromaffin cells of the adrenal gland, and the neurons and glia of all three peripheral nervous systems (sensory, autonomic, and enteric) (Le Douarin and Kalcheim, 1999).

Figure 1. Neural crest-like cells giving rise to pigment cells have been found in urochordates but not in cephalochordates.
It has been suggested that the formation of the neural crest was a crucial step in vertebrate evolution, facilitating the switch from a passive (e.g. filtering) to an active feeding by the formation of jaws and other head structures (Northcutt, 2005). Recent data showed that a cell population very similar to the NC produces the pigmentation of the Caribbean tunicate *Ecteinascidia turbinata* (Fig. 1) which means that this function and the origin of a cell population related to neural crest can be dated back as early as the urochordates (Jeffery et al., 2004; Meulemans, 2005). Traditionally urochordates have been described as a separate lineage that budded off from the vertebrate tree prior to the formation of cephalochordates and vertebrates, but the existence of a neural crest like cells in urochordates suggests that it might be the other way around since cephalochordates lack these cells (Graham, 2004; Jeffery et al., 2004). This is an example of how developmental biology can impinge upon our understanding of evolution.

### 2.1.2 Model systems and techniques

After being originally described in the chick, neural crest research was mostly carried out in the amphibian *Xenopus laevis*. However, the development of the chick–quail chimera system, in which donor quail cells are accepted and integrated into a chick host, changed the primary model system into that of avians. This technique, based on the easy access to the embryo and its great ability to withstand even major surgical manipulations, offered an unsurpassed opportunity to follow the fate of cells over a much longer period of time than when using dyes. The method was originally based on discrimination between chick and quail cells by their nuclear size and morphology in sections. Chick nuclei have its heterochromatin spread in several small chromocentres while the quail nuclei are very conspicuous due to one large mass of heterochromatin in the centre (Le Douarin, 1970). This characterization was the topic of many studies but with the development of species specific antibodies the procedure was made even simpler. The antibody preferred is the monoclonal QPNC (quail non-chick perinuclear antigen) raised by Carlson and Carlson and it is still today available from Developmental Studies Hybridoma Bank (DSHB). These two species however, do not lend themselves to genetic manipulation and the experimenter is, on the molecular level, limited to gain or loss of function experiments via injected mRNA, cDNA or siRNA. The entrance of modern molecular techniques genetically modifying organisms has led to the employment of a third major model system; the mouse. The possibility to manipulate the genome of the mouse has revolutionized biological sciences and the neural crest field is without exception. The majority of neural crest work is still being performed in the chick which is why we, in spite of its limitations, understand the different processes better in the avian system. I think that the field eventually will benefit from using the mouse. Another model system that has gained increasing interest lately is the zebra fish *Danio rerio*. This is due to the ease at which one can manipulate the expression of different
genes, together with the transparency of the animal and the short generation time of 3-4 months. The four organisms differ in some aspects, especially on molecular level, but in general share many features. The model organism utilized in this thesis work is the house mouse *Mus musculus*.

### 2.1.3 Induction and migration

The neural crest arises after gastrulation in the neural plate border between the epiderm and the neural plate. The induction of neural crest is accomplished in slightly different ways in the different species, but the common denominator in all species is the signalling of Notch, BMPs, Fgfs and Wnts (Meulemans and Bronner-Fraser, 2004) turning on early neural crest markers such as Msx1, FoxD3, snail, slug, pax3, and AP2. This specification occurs at the time of neural tube closure, when the initial neural plate becomes the neural tube (later to become the CNS). The cells that are specified to a neural crest fate are embedded in the dorsal neural tube at this time. After induction of the neural crest, and in order to be able to migrate away, the cells have to change from being part of highly structured epithelia like the dorsal neural tube. This is achieved during what is termed an epithelial to mesenchymal transition (EMT) which involves the down regulation of cell adhesion molecules like N-CAM and N-cadherin and upregulation of others like cadherin-7, a marker for neural crest cells (Nakagawa and Takeichi, 1998). In addition to releasing the moorings to their neighbors the cells also secrete proteases, which are able to break down the bond between other cells in their migratory path, thereby increasing their mobility (Valinsky and Le Douarin, 1985). Neural crest cells migrate in waves along defined pathways when they leave the neural tube. There are different pathways for the cell to migrate along (Fig. 2), which one a particular cell is going along is dependent on when in time the cell commences its migration. The first wave to exit the tube will migrate ventromedially through the anterior sclerotome into the area of the dorsal aorta and give rise to the sympathetic chain and the chromaffin cells of the adrenal cortex (Le Douarin and Teillet, 1974). Once this is accomplished the following cells in the wave will arrest their migration around the level of the neural tube to give rise to the dorsal root ganglia along with the boundary caps. The second wave to follow takes a dorso-lateral path between the dermamyotome and the skin –these cells will migrate throughout the extent of the embryo and give rise to the pigmentation of the animal.

### 2.1.4 Neural crest fate vs. potential

**Potential on population level**

The amazing multipotency of the neural crest makes it a powerful tool for unraveling molecular and cellular cues for specification of different cell types. What are the signals acting on the neural crest cells, when and where are they specified? One major difference within the neural crest population is seen along
the rostro-caudal axis. Neural crest from the head region gives rise to bone and cartilage, while crest cells from sacral and vagal regions give rise to enteric neurons and those in the trunk give rise to sympatho-adrenal cells. This rostro-caudal layout reflects the fate of the cells and has been determined by extensive tracing studies constructing a fate map of the neural crest (Le Douarin and Teillet, 1971; Le Douarin and Teillet, 1973). This is however different from the potential of the cells, which appears to be the same along the rostro-caudal axis. Experiments to investigate potential include in vitro differentiation and heterotopic grafting. For example, trunk neural crest was shown to be capable of giving rise to cartilage and bone when cultured in vitro and also when grafted into the head region (McGonnell and Graham, 2002). Furthermore, heterotopic grafting of trunk cells, normally giving rise to sympatho-adrenal cells, into the vagal crest showed that these could migrate correctly and give rise to enteric neurons (Nakamura and Ayer-le Lievre, 1982) and neural crest cells from all levels are able to form SA-cells (Le Douarin, 1986). Trunk neural crest has in addition been shown to have odontogenic potential (Lumsden, 1988). The above studies were all performed on a multicellular level and did not address the potential of individual cells.

**Trunk neural crest derivatives**

- 1st Ventromedial migratory pathway
  - Autonomic nervous system
    - Sympathetic chain (1)
    - Enteric nervous system (2)
    - Sensory nervous system
    - Dorsal Root Ganglia (3)
    - MEP Boundary cap (5)
- Dorsolateral migratory pathway
  - Pigment cells of skin (4)
- 2nd Ventromedial migratory pathway
  - DREZ Boundary cap (6)

![Diagram of neural crest migration pathways](image)

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Figure 2: Migration pathways of the neural crest cells.

First wave of cells (long red arrow) will migrate ventrally through the rostral portion of the somite to colonize the gut and form the sympathetic chain. Later cells in the same wave will give rise to the boundary cap of the motor exit points (MEP) and the sensory neurons of the dorsal root ganglia. The second wave will follow the dorso-lateral pathway (green arrow) and give rise to the melanocytes of the skin. The last cells to migrate away are the ones giving rise to the boundary cap of the dorsal root entry zone (DREZ).
Clonal analysis of potential

The potential of individual cells can be addressed by either clonal cultures or by tracing of individual cells in vivo. Neural crest cells can be isolated by plating whole neural tubes for 24 hours, allowing for neural crest migration onto the plate and subsequent removal of the tube (Cohen and Konigsberg, 1975). For clonal analysis the neural crest that still remained in the plate can be diluted and replated onto a layer of feeder cells. Several studies have shown the heterogeneity of migrating neural crest, identifying between three and 21 different populations of migrating neural crest cells (Baroffio et al., 1991; Sieber-Blum, 1989a; Sieber-Blum and Cohen, 1980). Tracing individual cells in mass cultures (where the neural tube is allowed to stay in the culture) is another strategy. It was shown that, if marked immediately after leaving the neural tube approximately 50% of the migrating cells gave rise to homogenic clones producing either glia, neurons or melanocytes (Henion and Weston, 1997). Another technique less vulnerable for changes induced by culture conditions or the selective survival/death of plated cells is that of in vivo tracing of individual cells. This can be accomplished using either a dye targeting specific cells (with the risk of dilution in each division) or using viruses infecting cells at random (Frank and Sanes, 1991). Using these techniques it was evident that a single neural crest cell would often give rise to both neurons and glia and that it could contribute to both the sympatho-adrenal lineage simultaneous to the sensory (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Some years later markers identifying two distinct populations expressing either tropomyosin receptor kinase receptor C (TrkC) or c-Kit were identified, in which TrkC$^+$ cells would give rise to glial and neuronal colonies while c-kit$^+$ would only contain melanocytes (Luo et al., 2003).

The neural crest stem cell

The finding of particularly one of the above mentioned neural crest cell types proved very important, namely the multipotent one. This cell type could, according to Le Douarin and colleagues give rise to all major lineages of the neural crest, but occurred only in 1 out of 350 cells (Baroffio et al., 1991). This population of cells was however enriched by fluorescence-activated cell sorting (FACS), using antibodies directed against low affinity nerve growth factor receptor (also known as p75) and further characterized by the Anderson lab (Stemple and Anderson, 1992) who claimed that 25% of the neural crest cells were actually multipotent. An important finding was that after subcloning, the p75$^+$ cells could still give rise to multipotent progeny, a trait however that only lasted for a couple of cell divisions. This finding indicating that the cells had stem cells properties alas with a limited capacity to self renew. Another attempt to find cells that retained their potential after the cessation of migration led to the discovery that the sciatic nerve from embryonic day (E) 14 not only contained Schwann cells (marked by the peripheral myelin protein P$_{0}$) but also a P$_{0}$ negative p75 positive population that had the stem
cells characteristics sought after (Morrison et al., 1999). They showed that this population was multipotent during a short time window, and that the ability to form neurons decreased drastically after only one day of development of the animal. One caveat however, was that these cells could not be propagated or kept for more than 10 days in culture without losing multipotency. This cell type was named neural crest stem cell (NCSC). It was not long until several structures containing NCSCs was identified including the adult gut (Kruger et al., 2002). There are some discrepancies regarding nomenclature in the field. From here on I define, post migratory multipotent cells with the ability to self renew, residing within neural crest derived structures as NCSCs. This is in contrast to still migrating neural crest cells with stem cell properties, which will be named neural crest cells (NCCs). The identification and isolation of NCSCs meant that here was a tool available for the studying of directed differentiation of neural crest cells.

**Differentiation of NCCs and NCSCs**

The first study using the technique of enrichment identified glial growth factor (GGF) as directing neural crest differentiation towards a glial fate (Shah et al., 1994) (this will be discussed further in conjunction with paper II). Another early study showed the involvement of retinoic acid in the development of melanocytes and adrenergic lineages from neural crest cells in a clonal assay on migrating neural crest cells (Dupin and Le Douarin, 1995). Several factors have been identified since using the NCSCs, among them transforming growth factor β (TGFβ) inducing smooth muscle differentiation while different concentrations of BMPs instructed towards a neuronal phenotype. A lot of effort was put into elucidating the choice between a glial and a neuronal fate. Except BMPs and neuregulins, another signalling pathways was identified, Notch (Morrison et al., 2000). In addition to extracellular factors several genes involved in neurogenesis were identified, among them members of the basic helix loop helix (bHLH) family such as the neurogenins (NGNs) 1 and 2, Phox2b, Pea3, Erm and Mash1 (Lo et al., 1999; Lo et al., 1998; Lo et al., 1991; Ma et al., 1999; Ma et al., 1996; Paratore et al., 2002). As mentioned before the neural crest gives rise to two major types of neurons; sensory and autonomic, which share some markers but can be distinguished by others. They all express neuronal markers like βIII-tubulin, and peripherin (a preferentially peripheral marker) while they rely on different sets of proneural or subtype specific genes in their differentiation. NGNs, Brn3a, TrkA, Pea3, Er81, are involved in sensory genesis (see section 2.7.1) and mash1, phox2b, and dHand are involved in autonomic differentiation (Howard et al., 2000). Interestingly, all data on sensory genesis was hitherto derived from studies of NCCs since the only peripheral neurons that could be obtained from NCSCs were of the autonomic lineage. Additionally these could only be grown using medium containing chick embryo extract (CEE) making analysis of soluble factors less defined. This was the starting point of my thesis work.
3 THE PAPERS OF THE THESIS

3.1 PAPER I: BOUNDARY CAP NEURAL CREST STEM CELLS

The initial objective: Isolate a NCSC capable of differentiation into sensory neurons. We hypothesized that if there was going to exist such a cell, chances would be that it resided among the sensory neurons. Hence the initial strategy was clear; dissect dorsal root ganglia of different stages and try to propagate any stem cell using a defined medium. Ziller et al had in two papers during the 80’s shown that sensory neuron precursors could be grown for some days in defined medium, and that adding CEE inhibited generation of sensory neurons while it supported autonomic differentiation in neural crest cultures (Ziller et al., 1983; Ziller et al., 1987). Additionally Anderson’s lab had found that any addition of fetal bovine serum (FBS) to the medium immediately caused all stem cells to differentiate and the cells to lose their p75 expression (Stemple and Anderson, 1992), a finding that we later would confirm using our stem cells. After struggling with cell densities, plate coatings and media supplements we could observe the formation of p75 and nestin positive cell clusters in the plate. These clusters would grow until they detached from the plate, due to mechanical shearing, forming neurospheres, a well known feature of neuronal stem cells. We had found something that could be a NCSC! We were able to propagate the cells seemingly without limitations, and the early clones were kept in the lab for more then 8 months with weekly subcloning. This is a feature similar to that of neural stem cells derived from the CNS but not described in NCSCs previously. Characterizing the cells we could confirm the differentiation into peripherin positive cells with extensive neurites (suggesting a peripheral phenotype) and reverse transcriptase polymerase chain reaction (rtPCR) pointed at a sensory phenotype with both NGN 1 and 2 being expressed. At this point we also controlled for and could exclude spinal cord contamination by including a number of central markers that one could suspect that the cells would express if they were derived from the CNS (Otx1, Pax2, and Pax5). A finding that puzzled us was the strong expression of zinc-finger transcription factor Krox20 by the stem cells. Being a marker for mature Schwann cells it greatly surprised us since we could see no signs of Schwann cell differentiation among the cells (Topilko et al., 1994). Occasional GFAP positive cells were seen, a sign of partial differentiation within larger spheres, but previous reports claimed that krox20 expression does not arise in culture before 18 days of directed differentiation in vitro (Langford et al., 1988). It seemed unlikely that there was differentiation to that extent occurring. The finding of Krox20 however turned out to be of great importance since this is also a marker for a population of cells called the boundary cap cells (Wilkinson et al., 1989). I will make a short break in the story for an introduction of this structure that would occupy most of my thesis work.
3.1.1 The boundary cap

The boundary cap (BC) cells of the spinal ganglia were initially identified as a population of neural crest derived cells that migrated down to prospective entry and exit points of motor and sensory neuron efferents going between the peripheral and central nervous systems (Altman and Bayer, 1984). These cells find their position already at E13 in the rat and around E10.5 in the mouse (Altman and Bayer, 1984; Topilko et al., 1994) which is prior to axonal out or ingrowth. The signal locating the cells at the prospective exit/entry points is unknown but experiments on chick hindbrain BC cells, where neural crest from rhombomere (r) 3 and r5 was substituted ectopically with that from r2 and r4 without creating additional BCs, suggest that there is a localization signal derived from the neural tube epithelium (Niederlander and Lumsden, 1996). The fact that migrating neural crest cells secrete proteases has caused speculation whether the BC cells can cause the opening of the basal lamina at the entry/exit points but no evidence for this exists. The BC cells remain at the location of the dorsal root entry zones (DREZ) and motor neuron exit points (MEP) of the DRGs throughout development until the time of birth in rat (Golding and Cohen, 1997). The interphase between the central and peripheral nervous system at the DREZ change just after birth with astrocytes protruding out from the CNS into the roots as shown by experiments on cats (Berthold and Carlstedt, 1977). This is thought to infer strength in the roots but astrocytes have also been implicated in blocking axonal regeneration with the astrocytic expression of growth inhibitory molecules (Beggah et al., 2005; Livesey and Fraher, 1992; Ramer et al., 2001). One interesting feature of the late BC is that the cells seem to block astrocytes protruding out form the CNS (Golding and Cohen, 1997).

In the recent years the BC has received increased attention with two very elegant studies spurring the interest. The first investigated the role of the boundary cap cells in retaining spinal cord integrity at the CNS/PNS border of the ventral MEP (Vermeren et al., 2003). They showed using several techniques; splotch mutant (pax3 -/-) lacking neural crest, mechanical ablation of neural crest, or the exquisite technique of Krox20 directed diphtheria toxin ablation of the BC, that removing the ventral boundary cap resulted in central motor neurons migrating out into the periphery. This effect of migration could be rescued by the heterologous grafting of neural crest cells. The signal keeping the motor neurons in the CNS still remains to be found. The second study was about a role of the BC that more closely relates to my present work. It was inspired by a much earlier finding, using tritiated thymidine pulses that the BC continues to divide during the entire embryogenesis (Altman and Bayer, 1984). This, together with the fact that its disappearance could not be accounted for by apoptosis, suggested that the cells migrated away (Golding and Cohen, 1997). Maro et al. utilized the technique of lineage tracing Krox20 expressing cells in a series of experiments to show that the cells of the DREZ
boundary cap indeed migrated into the DRG (Maro et al., 2004). In the DRG the BC cells gave rise to satellite cells, sensory neurons (preferentially nociceptive TrkA expressing neurons) but they also gave rise to the Schwann cells of dorsal root. The only caveat of this approach is that at E15 in the mouse all Schwann cells turn on Krox20 expression in their normal differentiation process (discussed in paper II) thereby rendering the study of the fates of the cells after this time point impossible. After this report several groups working on neural crest and DRG development have directed some attention to this group of cells, leading to the discovery of markers expressed. Before this few markers where known, except for Krox20. One is mono amino oxidase B (Maob) that had been described in BCs of cranial nerves (Vitalis et al., 2003) (a finding we utilized in paper I). Among recently identified markers are the chemokine receptor Cxcr4 which binds stromal cell-derived factor 1 (SDF1) and has been implicated in migration of neural crest cells seem to be expressed in the BC (Belmadani et al., 2005). mRNA from the gene Lgi4, whose mutation is responsible for the claw paw phenotype, due to lack of myelinisation, was also heavily expressed in the BC at E14, a fact that may result from glial differentiation (Bermingham et al., 2006). Another marker not previously described in the BC per se, but that has the potential to not only be a BC marker but also fate neural crest cells to a BC fate, is GDF7. Using Gdf7-cre driven lineage tracing it was shown that these cells leave the dorsal spinal cord late, are concentrated to the DREZ and dorsal root of the DRG and that they give rise to predominantly TrkA positive neurons within the DRG (Lo et al., 2005). Given that this is the hallmark for DREZ BC cells it is remarkable that the authors did not address the cells relationship to the BC. Additional markers, stage specific embryonic antigen 1 (SSEA-1) and high mobility group transcription factor Sox10, will be discussed in paper III and II respectively. But for now I return to the story of Paper I.

3.1.2 Sensory neuron stem cells
We first set out to confirm the BC identity of the cells. For this we have two different lines of evidence. The first was a rather direct method: micro dissection of the boundary cap avoiding the bulk of the DRG. Using this technique allowed us to separately culture cells from the DRG and the dorsal BC confirming at least tenfold numbers of stem cells in the BC part of the dissection. This has later been confirmed for ventral BC cells from the MEP (data not shown). In another, much less direct, line of evidence we utilized the expression of Maob by the cells along with an enrichment step occurring on day two of culturing. We could see that the BC cells were enriched during this step and that it was not due to proliferation of cells or induction of the marker. After confirming multipotency by clonality experiments (showing that the cells could give rise to neurons, glia and smooth muscle cells) we decided to name the cells boundary cap neural crest stem cells (bNCSCs). The initial question however still remained: could these cells, in
contrast to previously described stem cells produce sensory neurons? Using the FoxS1 (named Fkh3 in paper I) mouse constructed by Anna Cederberg in the Enerbäck lab at Gothenburg University we could confirm differentiation of early sensory neurons. FoxS1 is a transcription factor of the forkhead family and we showed the exclusive expression in sensory ganglia during development (Fig. 3). Using this marker together with a marker of more mature sensory (nociceptive) neurons like calcitonin gene related peptide (CGRP), we could confirm the formation of sensory neurons in culture.

We also confirmed the physiological function of these sensory neurons using the technique of calcium imaging, a technique based on the fact that neurons respond by changes in intracellular calcium concentration in response to stimuli. By measuring these calcium transients one can elucidate which stimulus a cells respond to. We used stimuli typical for sensory neurons (discussed in detail in Paper IV); capsaicin (the “hot” substance in the chili fruit) that has been shown to activate heat responsive nociceptive neurons (Caterina et al., 1997) and menthol activating neurons detecting an innocuous cool temperature (McKemy et al., 2002; Peier et al., 2002). We also used a cold stimulus, a broader stimuli exciting both cool and cold neurons, and hypoosmolarity causing a cell swelling that will activate stretch receptors in putatively mechanosensitive neurons (Viana et al., 2001). Using these stimuli we could conclude several things: 1) the cells do not only express some markers for sensory neurons but are actually functional. 2) Several different neurons could be derived from a single bNCSC indicating that specification of subtype occurs after migration from the BC. 3) In culture, in contrast to the in vivo findings where the vast majority was TrkA positive nociceptors (Maro et al., 2004), it seems that not only nociceptive neurons are born.

Figure 3. (A) Whole-mount in staining of a Foxs1lacZ/ mouse for β-gal at E11.5, shows the highly restricted β-gal expression to cranial and spinal sensory ganglia (schematic in B). (C) β-gal expression in a differentiated stem cell clone from the same mouse confirms the presence of sensory neurons in the culture. Scale bars: 1 mm in A, 100 μm in B.
This is a finding supported by our subsequent but unpublished observation of markers like receptors TrkB, TrkC and the phosphorylated form of neurofilament 200 (recognized by the RT97 antibody) in neurons derived form bNCSs. The discrepancy could suggest a signal being present within the DRG directing the cells towards a nociceptive fate upon entering the ganglia. Since the nociceptive neurons are the small diameter population being the last of the sensory subtypes to be born (Lawson and Biscoe, 1979), it is not unlikely to think that the nociceptive signal lingers directing the new BC derived cell to a nociceptive phenotype. In previous cultures of NCSCs only autonomic differentiation had been observed (Morrison et al., 1999; White et al., 2001). The question, whether obtaining sensory neurons was merely due to differences in culture conditions or if it reflected a true difference in potential to previous NCSCs remained. By isolating the NCSCs from the sciatic nerve and showing that these failed to differentiate into sensory neurons we showed that it was due to a true difference in potential. We however failed to induce autonomic neurons both with the bNCSCs or those from the sciatic nerve. This shows that our culture conditions are not permissive for autonomic differentiation and thus the question whether bNCSCs have a broader potential than those from the sciatic nerve being able to form both neuronal lineages remains unanswered.

3.2 PAPER II: MYELINATING SCHWANN CELLS FROM bNCSCs

One major issue in neuronal regeneration after injuries is to overcome the non-permissive environment created by endogenous cells in response to the injury. Peripheral glial cells, Schwann cells (SCs), have attracted special interest since they have been shown to create a supportive environment for regrowth (Stangel and Hartung, 2002) and to myelinate regrowing axons, restoring conductance velocities (Honmou et al., 1996). The clinical use of SCs today is however hampered because of the limited availability. We set out to test whether the bNCSCs could constitute a novel source of SCs, and to see whether these cells were able to myelinate regrowing axons.

3.2.1 Schwann cell development

Schwann cells (SCs), along with peripheral neurons, are derived from the neural crest. Their development is characterized by the differentiation of discrete steps that exist during a prolonged time during embryogenesis. When axons of developing nerves are finding their way through the body they are accompanied by SC precursors around E12. These precursors are however not necessary for the path finding but for the survival of the axons once they have reached their targets since axons are created in normal numbers but subsequently die in animal models lacking peripheral glia (Britsch et al., 2001; Riethmacher et al., 1997). Once the nerve has reached its target (around E18) there will be the formation of a
perineurium, and at the same time the SC precursors will differentiate into immature SCs. These immature SCs encompass several axons at a time together with other immature SCs; and it is not until 3 days later that they will become mature SCs and start the myelinization process of a single axon. During these three days the presence of anti-myelinisation programs, like the Jnk-pathway, Sox2 and Pax3 expression, inhibits the progression onto the final step (Kioussi et al., 1995; Le et al., 2005; Parkinson et al., 2004). The down-regulation of expression of Sox2 and Pax3 and the inactivation of the Jnk pathway is dependent on Krox20 signalling, along with a number of other pro-myelinization transcriptions factors, and is the starting signal for myelinization (Parkinson et al., 2004). This is in accordance with our finding of Sox2 expression in all developmental levels, including bNCSCs, except that of the mature SCs. Other markers investigated by us, that had previously been described in the maturation process were; S100, Sox10, GFAP, fibronectin (FBn) and Krox20. S100 is a marker used to identify SC differentiation from stem or neural crest cells (Morrison et al., 2000). We could confirm S100 expression levels of differentiation except in undifferentiated neural crest cells. GFAP is turned on at E12.5 in the distal part of the spinal nerves that might be the first immature SCs and in adulthood it was expressed in all peripheral glia (SCs and satellite cells). It is however expressed in higher levels in non-myelinating SCs than in the myelinating. The final step from immature SCs was accompanied by the loss of Sox2 expression and the acquirement of Krox20 in myelinating cells, which is in line with previously discussed findings. Another marker used in this study is myelin basic protein (MBP), a major component of myelin, which stains already myelinated fibers.

3.2.2 Myelinating Schwann cells from bNCSCs

Differentiation into Schwann cells
Other NCSCs have been shown to differentiate into Schwann cells, using markers such as GFAP, S100 and c-neu, in the presence of neuregulins but have not been assayed for the formation of mature SCs. A population of our bNCSCs spontaneously differentiated into GFAP positive glial cells but failed to differentiate into mature Schwann cells without the addition of neuregulins. However with the addition of neuregulin broad sheets of Schwann cells were seen in cultures. Neuregulins has been shown to act as mitogens on Schwann cell precursors but not on NCSCs (Lemke and Brockes, 1984; Shah et al., 1994) and is intimately involved in most processes in Schwann cell development, proliferation of precursors to the exact matching of SCs to axons by selection (Garratt, 2000). This is in accordance to our cultures where the increase in proliferation, creating the sheets of cells, only commences after a time of differentiation and the onset of S100 expression. These sheets of SCs exhibited typical morphology stretched cell bodies in pavement like arrays with elongated blunt ended nuclei (Jessen and Mirsky, 2002). bNCSCs directly grafted into an intact sciatic nerve failed to
differentiate into SCs indicating that the signals necessary were not present. This is not surprising since in the adult intact nerve the SCs have established an autocrine survival loop and no longer depend on secreted neuregulins for their survival (Meier et al., 1999). The low concentration or lack of neuregulins could explain the failure of the stem cells to differentiate into S100+ cells.

**Myelinization by the bNCSC derived Schwann cells**

In order to see whether the SCs not only expressed the right markers but also could myelinate axons we studied cocultures with explanted DRGs. In this model we observed thick bundles of nerves that defasciculated as they reached the area of bNCSC derived SCs. MBP staining confirmed the first finding of in vitro myelinisation by NCSCs. Their ability to myelinate was then tested in vivo, by grafting of pre-differentiated SCs in plastic tubing into severed nerves. The pre-differentiated SCs survived and we observed myelinating MBP+ SCs in the tubing. We never saw any grafted cell outside the tube, indicating that there is no significant migration of grafted cells along the nerve. Nor did we observe any signs of tumors or extensive cell proliferation during the 90 days of grafting. These data together suggests a clinical relevance and could contribute to the development of new cell-based therapeutic strategies for nervous system repair.

### 3.3 PAPER III: STEM CELL CONTROL BY GABA

In the previous sections I have showed that the bNCSCs can give rise to functional sensory neurons and ensheathing Schwann cells. Several questions remain about the life of the stem cells within the boundary cap. One major question is: why do the BC cells have stem cell properties? We and others have speculated that it could constitute a kind of cell depot or spare part store to buffer and correct mistakes during development (Hjerling-Leffler et al., 2005; Maro et al., 2004). If the function of the BC is to buffer developmental deviations in the DRG there has to be some sort of retrograde signal from the target tissue to specify how many cells that needs recruiting from the BC.

#### 3.3.1 SSEA-1 labels multipotent BC cells

Since before the transcription factor Krox20 and the mitochondrial flavoprotein Maob had been described as markers for BC cells (Wilkinson et al., 1989; Vitalis et al., 2003). We were interested in identifying additional markers, and therefore looked at known markers for multipotent cells: Sox10 and SSEA-1. In paper II we showed that dissociated bNCSCs were positive for Sox10, and in this paper confirmed that the BC cells also expressed this multipotency/glial marker. SSEA-1 (also known as LeX-antigen, CD15, trisaccharide 3- fucosyl-N-acetyl-lactosamine or FAL) has been described as a marker for neuroblasts and a subset of more mature neurons in the sensory lineage (Jessell and Dodd, 1985; Sieber-Blum,
1989b). It was first identified by raising antibodies against F9-teratocarcinoma cell line and was initially shown to be expressed by 8 cell stage embryos of the mouse (Solter and Knowles, 1978). It has, since the late eighties, been used as a marker for fluorescent flow cytometry in the field of immunology (Ohmori et al., 1989). In 2002 it was successfully used to sort a population of neural stem cells from the adult SVZ (Capela and Temple, 2002). We find that BC cells, and cells migrating from the BC, express this marker (Fig. 4), specifically in the time window when the BC cells have been shown to be stem cells in Paper I and give rise to neuronal progeny in vivo (Maro et al., 2004). Because SSEA-1 is a cell surface marker, this opens up for the possibility to isolate the BC cells using fluorescent activated cell sorting (FACS).

3.3.2 Stem cell regulation
Stem cells reside within a tissue and can contribute to either the tissue where it resides or, sometime after extensive migration, another tissue further away (e.g. in the case of SVZ stem cells migrating to the olfactory bulb). Tight control of stem cell proliferation during development and in the adult is essential to avoid developmental defects and diseases such as cancer. Signals acting on stem cells, negatively or positively regulating their proliferation, can either be local (autocrine or dependent on cell to cell contact) or diffusible. Both kinds of signals may be present at the same time; local signaling regulating stem cell niche size, and other conveying how much cells are needed in the target organ. The latter signal does not necessarily have to affect proliferation since, in the presence of a local auto-regulation of stem cell niche population size; it would suffice with a signal regulating either emigration from the niche or differentiation of stem cells into post-mitotic progeny. Another factor to account for is where in the lineage of differentiation the signal acts. Is it on the actual stem cells or on more restricted but still dividing precursors? While the different populations in the adult and developing brain are well known (Sommer and Rao, 2002), much less is known about what happens after the boundary cap stem cells have reached the ganglion. Although we do not know definitely whether these cells still proliferate, data from tritiated thymidine studies suggests that they do not proliferate extensively once inside the ganglia (Altman and Bayer, 1984).

There are several neural stem cell niches described in the mouse; the subventricular zone (SVZ), the subgranular zone (SBZ), within the enteric nervous system and the developing sciatic nerve (Alvarez-Buylla and Lim, 2004; Kruger et al., 2002; Stemple and Anderson, 1992). In experiments designed to elucidate the plasticity of stem cell contribution (how much the contribution with stem cells within a tissue can increase in response to an injury), a remarkable ability of the adult brain to respond to the depletion of progenitors was seen (Doetsch et al., 1999a; Doetsch et al., 1999b). This suggests that stem cells of the SVZ have a significant capacity for plasticity regulated by cell extrinsic signals to control that the appropriate
amount of cells is present. In a study in which the olfactory bulb (OB), the distant target tissue for the neuroblasts produced in the SVZ, was removed no evidence for long range signals regulating proliferation or migration emanating from the OB were found (Kirschenbaum et al., 1999).

Positive regulation of proliferation

The stem cell niches in the brain are intimately connected to blood vessels, and vascular endothelial cells themselves have been implicated in proliferation and differentiation of stem cells in the adult brain (Palmer et al., 2000).

Figure 4. Gradual loss of BC and multipotency markers during migration. The BC cells express all four markers; Krox20, Maob, SSEA-1 and Sox10 before they migrate away from the DREZ. On their way into the ganglion it appears that they down-regulate the different markers sequentially. I show GABA acting on bNCSCs and propose the same signal to be present in vivo regulating proliferation.
This suggests that vascularization is a process intimately connected to stem cell regulation, a finding confirmed in the songbird where BDNF from endothelial cells induces progenitor proliferation (Louissaint et al., 2002). Also, exogenous applied BDNF have the same effect in the rat (Benraiss et al., 2001). Endothelial cells release several soluble factors of which 27 have been described as potential regulators of neighboring cells (Rak et al., 1996). Some of the first molecules investigated were basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) receptor ligands. These are the primary mitogens used for in vitro proliferation of stem cells from all neuronal niches (also for bNCSCs and other NCSCs, see paper 1). Stem cells and progenitor in the brain express the receptors (Morshead et al., 1994) and introducing these factors in vivo increases the rate of neurogenesis (Craig et al., 1996; Doetsch et al., 2002). Vascular endothelial growth factor (VEGF) has also been shown to have a stimulating effect on stem cell proliferation (Zhu et al., 2003), but is also secreted by the stem cells stimulating vascularization (Breier et al., 1992). However in a recent study the maintenance of stemness and proliferation of SVZ stem cells was shown to be the result of a soluble factor other than the ones previously mentioned, which failed to completely mimic the effects of cocultures with endothelial cells (Shen et al., 2004). Very recent data identify this factor as pigment epithelial-derived factor (PEDF) (Ramirez-Castillejo, 2006), a molecule that in vitro mimics the effects of endothelial co-cultures and that acts specifically on the stem cells and not on progenitors.

In addition to the above findings the list of soluble molecules found to affect neurogenesis grows; Wnt1, Wnt3, TGFα, CCg, IGF1, amphiregulin, sonic hedgehog, estrogen, NT3 and 5-HT all act by increasing proliferation on progenitors or stem cells in the CNS (Aberg et al., 2000; Banasr et al., 2001; Falk, 2002; Lai et al., 2003; Lie et al., 2005; Panhuysen et al., 2004; Tanapat et al., 1999; Taupin et al., 2000; Tropepe et al., 1997; Zhu et al., 2003). Much less is known about membrane bound molecules increasing stem cell proliferation. One such molecule, that has been extensively studied, is Notch which has been shown to be involved in a number of processes during development. Its pro-proliferative effects on stem cells are mediated through the down regulation of proneural bHLH genes via the activation of Hes1 and Hes5 (Bertrand et al., 2002; Hitoshi et al., 2002).

Negative regulation of proliferation
In order to avoid excessive expansion of stem cells, something that hypothetically could lead to cancer or other defects, a signal acting like a brake to decrease proliferation would be required. Much fewer signals have however been found to reduce stem cell proliferation, but there are some. Recently, endogenous EphA7 acting through Ephrin-A2 has been shown in vivo to negatively regulate both the proliferation of stem cell cultures as well as the fast dividing progenitor pool in adult SVZ (Holmberg et al., 2005). Glutamate agonist (both NMDAR and Non-NMDAR), nicotine (agonist for nicotinergic acetyl choline receptors) and the
neuropeptide PACAP has been shown to negatively regulate proliferation in neural progenitor cultures from developing rat cortex or dentate gyros (Cameron et al., 1998). Another transmitter molecule that has been implemented in negatively regulating stem cell or progenitor cells is gamma amino butyric acid (GABA), a molecule which we chose to study within the boundary cap stem cell niche.

### 3.3.3 Effects of GABA signalling

GABA is the most abundant inhibitory neurotransmitter substance within the central nervous system being responsible for the activity of the inhibitory interneurons via synaptic release. Another effect of GABA has been shown during development, to decrease cell division in neuronal precursors within the CNS (LoTurco et al., 1995; Owens and Kriegstein, 2002). Additionally, GABA was recently shown to act negatively on proliferation of adult subventricular GFAP\(^+\) stem cells via the chloride permeant GABA\(_\text{A}\) receptor (GABA\(_\text{A}R\)) (Liu et al., 2005). This non-synaptic calcium\(^{2+}\)-dependent signaling was shown to originate from spontaneous activity of the neighboring neuroblast which, in contrast to the GFAP\(^+\) cells, expressed GABA. We found that the BC cells not only were immunoreactive for both the GABA synthesizing enzyme GAD65/67 but also for the GABA transporter VGAT. This expression was only found during the time window when the BC contributes with both neurons and glia. In order to begin investigating whether GABA controls the BC cells, I turned to our in vitro culture system. The bNCSCs expressed several subunits of the GABA\(_\text{A}R\). In addition, application of GABA\(_\text{A}R\) specific agonist muscimol completely blocked EGF/bFGF stimulated proliferation in bNCSCs in a calcium\(^{2+}\) independent fashion, without affecting survival or differentiation. We also show the presence of endogenous production of GABA by the bNCSCs in EGF/bFGF containing cultures that can be blocked by bicuculline resulting in increased proliferation. This is in contrast to the finding in striatal PSA-NCAM\(^+\) positive precursors where EGF acts, at least in part, by decreasing GABA secretion and where addition of EGF abolishes all effects of GABA antagonists (Nguyen et al., 2003). This discrepancy or difference could be attributed to differences in cell density during the experiments rather than real intrinsic differences between cells.

Our findings are intriguing since the BC system appears to share important aspects with that of the adult SVZ but with some important exceptions. The GABA producing cells were not the stem cells in the SVZ, but rather their progeny. This is in line with the previous finding, in which depletion of neuroblasts increased the proliferation of GFAP\(^+\) stem cells (Doetsch et al., 1999b), possibly via a loss of GABA signalling. In our system however, there seems to exist an autoregulatory loop where the stem cells themselves regulate their proliferation. Furthermore, the signal from the neuroblasts in the SVZ was dependent on calcium\(^{2+}\) signalling within the cell subsequent to depolarization (Liu et al., 2005). Experimentally this depolarization of the neuroblasts was induced by either direct electrical stimuli or
via local increases of $K^+$. In our in vitro system the stem cells releasing GABA do not respond to $K^+$, which indicates a mechanism for GABA release in bNCSCs, different than the one previously described. Also, in contrast to progenitor cells of the developing cortex (LoTurco et al., 1995), the bNCSCs do not respond to GABA with increases in Ca$^{2+}$ levels. The fact that GABA has different roles in different progenitor/stem cell systems is further supported by the finding that the rapidly dividing type II cells of the SGZ, receives synaptic input from neurons of the hippocampus and that this input stimulates neuronal differentiation (Tozuka et al., 2005).

Given that the BC stem cell niche regulates its own size, these finding would argue that it would suffice with a signal from the target organ (DRG) regulating migration from the boundary cap. It is also possible that the GABA signal from the BC stem cells is not sufficient alone, and that the whole target tissue directly controls proliferation of the stem cells (Fig. 4). Whether this is the case or not remains to be investigated, as well the identity of such a secondary migratory signal. Interestingly, GABA has also been implemented in regulation of migration of stem cell derivatives from the SVZ (Bolteus and Bordey, 2004; Nguyen et al., 2003) and we show that synthesizing enzymes are expressed by developing DRG neurons at this stage.

3.3.4 Membrane potential, $K^+$, and proliferation

There are numerous reports showing that cells respond to electrical stimuli, with the two most obvious examples being neurons and muscle cells. A less studied cell type when it comes to membrane potential is the stem or progenitor cell. There are however, that describe how stem or progenitor cells respond to electrical stimuli or manipulations of membrane potential. One is that of the SVZ precursors releasing GABA in response to manipulations of membrane potential (Liu et al., 2005). Another is that of myofibroblasts changing their proliferative state, not in response to a ligand but, to a direct manipulation of membrane potential via $K^+$ levels (Chilton et al., 2005).

The membrane potential depends on three factors, differences in ion-concentration over the membrane, differences in ability of the selective ions to enter or leave the cell and third the transport of ions against their apparent gradient. One way of altering the membrane potential is manipulating the gating or open probability of $K^+$ permeable channels. The $K^+$ channel family is a very large family that recently was restructured and the different channels renamed (Gutman et al., 2003). In neurons some of these channels are involved in returning the cell to a resting potential via rectifying currents active in the later phase of the action potential, but are also involved in more long term regulation of membrane properties (Lesage, 2003). They have also been widely studied in the field of cardiology since they are responsible for the shape of the electrical waves of cardiomyocytes and are involved in many life threatening disease states (Rivera and
Lowes, 2005). Additionally, several of its members have been implicated in the regulation of cell proliferation in as diverse systems as cancer, blastocysts and leukocytes (Arcangeli, 2005; Price et al., 1989; Winston et al., 2004). In two of those system channels of the Merg family (also known as ERG1, ether a go-go related, LQT or Lqt2) have been described (Arcangeli, 2005; Winston et al., 2004). This family of channels, has also been described to be expressed by fetal CNS progenitors in the rat (Cai et al., 2004). The gene Kcnh2 encodes for the pore forming α-subunits of these channels and was identified in mammals ten years ago based on it homology to drosophila (Warmke and Ganetzky, 1994). We show that we can mimic the GABA response of the cells by blocking Merg using the antagonist cisapride. The cells express this channel and they decrease their proliferation if it is blocked. This might suggest that the action of GABA, instead of being specific with regards to intra cellular signalling, is to manipulate the membrane potential. If Merg is downstream of GABA\(_A\)R activation or just a parallel pathway remains to be elucidated. One way to address this is to try to affect the proliferation of bNCSCs by manipulation of K\(^+\) levels to see whether it is a true membrane potential effect. Another is to see what happens if we knock down the Merg channel using siRNA. If we can avoid an initial proliferation block it would be interesting to see if the effect of GABA is affected. Could this effect on membrane potential be common among stem, precursor and cancer cells? The effect of cisapride on cancer cells certainly suggests a link. Understanding this phenomenon could then be very beneficiary in developing treatment for developmental defects and cancer. In this paper we show that there at least exists regulation of BC stem cell proliferation in vitro and show histochemical data suggesting this to be the case also in vivo. The fundamental question however still remains: is there a retrograde signal from the DRG to the BC controlling plasticity in stem cell contribution, and could this signal be GABA?

3.4  PAPER IV: FUNCTIONAL DEVELOPMENT OF SENSORY NEURONS

3.4.1  Sensory neurons from neural crest cells
The specification of neural crest cells into different neuronal lineages has been studied extensively. One of the first findings was that BMPs promoted the differentiation of autonomic neurons in NCSCs. The signal promoting sensory differentiation remained for a long time elusive. Sensory neurons in the DRG are born in two distinct waves with large and medium diameter neurons being born first and small diameter neurons later (Lawson and Biscoe, 1979). Ma et al. identified the bHLH transcription factors NGN1 and NGN2 as involved in the different waves of sensory genesis (Ma et al., 1999). NGN2 is expressed already in migratory neural crest while NGN1 is turned on later after the migratory cells have entered the DRG anlagen. Neither is however crucial for sensory formation in general since in the NGN2 null mutant normal numbers of sensory neurons are
born, after some delay, suggesting redundancy between the two family members. In the NGN1 null mutant DRGs are formed but have a reduction in number of cells. In the double mutant the DRGs are absent (Ma et al., 1999). Another signalling system involved in sensory genesis is that of Notch. Notch has been shown to negatively regulate neuronal formation by instructing a glial fate in NCSCs (Morrison et al., 2000). The null mutant for Numb, a notch repressor, showed normal ganglion formation, i.e. neural crest migration, but no neuronal differentiation suggesting that blocking Notch signalling is crucial for sensory genesis (Zilian et al., 2001). The generation of autonomic neurons was unperturbed indicating a sensory neuronal specific function of Numb but not Notch. It was not until three years later in the lab of Lucas Sommer that the signal responsible for directing NCCs towards a sensory fate was identified (Lee et al., 2004). This group had previously demonstrated the necessity of β-catenin signalling in sensory genesis by specifically ablating its expression in neural crest cells, using a Wnt1 driven cre-lox system, leading to a complete loss of DRGs. In the later report they showed that β-catenin stabilization was enough to ectopically differentiate neural crest cells into sensory neurons at the expense of other fates, and that it could be accomplished by Wnt1 signalling in culture (Lee et al., 2004). Interestingly β-catenin stabilization did not lead to an increase in proliferation (as opposed to other stem cell systems, see above) but appeared to act exclusively in an instructive manner. Not all early neural crest cells differentiate into sensory neurons, some retain their multipotency, and thus a factor counteracting the effect of Wnt signalling must exist. In a study, BMP signalling was shown to counter act the activity of Wnt, suggesting a balance between the two pathways, leading to the very elegant hypothesis where high levels of Wnt leads to sensory differentiation while high levels of BMP induces autonomic, and the balance maintains stem cell properties (Kleber et al., 2005).

The differentiation of progenitors into differentiated progeny is accompanied by a drastic change in the transcriptional output. This is accomplished via the induction of transcription factors regulating gene expression. These transcription factors are a powerful tool as markers for different cell types and sensory neurons are no exception. The marker most used to show general sensory differentiation is the POU-domain factor Brn3a that was shown to be specific within the neural crest lineage, for sensory neurons (Fedtsova and Turner, 1995; Xiang et al., 1995).

3.4.2 Molecular specification of subtypes
Sensory neurons residing within the DRG are molecularly and functionally very heterogeneous group. No other neuronal population has been characterized to the same extent with regards to subpopulations. Division into subtypes has traditionally been made according to several criteria; cell size, neurotrophic dependency (and the corresponding expression of growth factor receptors/coreceptors; Trk and c-ret), axonal myelinisation and conductance velocity,
expression of neuropeptides, or binding of surface markers. More recently, insight into the molecular basis of subtype specification as well as function has been gained. With the recent findings of subtype controlling transcription factors of the Runx family and the emergence of TRP-channels as the principal ion channels determining the receptive properties of the peripheral terminals the field has taken several important steps on the path to understanding the formation of the somatosensory system.

**Trk receptors; development of subtypes**

Trk receptors were originally described as mediator of growth promoting and neurotrophic effects of a group of molecules called Neurotrophins (NGF, BDNF, NT3 and NT4/5). The limited availability of neurotrophins in the target tissue leads to an elimination of superfluous neurons that are unable to form correct connections and ensures the matching of amount of innervation to the size of the target. There are 4 different neurotrophins acting on three high affinity receptors present in sensory neurons of the DRG. These three Trk receptors in divide the sensory neurons into three groups corresponding extremely well with functionality: TrkA; marking small diameter, late born, unmyelinated (C-) fiber nociceptive neurons innervating the skin, deep somatic tissues and viscera; TrkB, mainly marking large to medium diameter, thinly or thickly myelinated cutaneous rapidly adapting mechanoreceptive neurons; and TrkC, marking large diameter proprioceptive neurons, with thickly myelinated fibers innervating Merkel cells, Golgi tendon organs, and muscle spindles, involved in the maintenance of body posture (Bibel and Barde, 2000). The Trk receptors are differentially expressed during development and the final pattern mentioned above does not match the initial expression; at early stages most cells express TrkC and many of them TrkB (Farinas et al., 1998; Kahane and Kalcheim, 1994; Snider and Wright, 1996). This is a transient expression and during specification a majority of the cells will turn off TrkC, forming TrkA, TrkB and TrkC positive discrete groups at late embryonic stages. The small diameter cells that will become C-fibers (80% of total cells) all express TrkA before birth but there is a perinatal switch in phenotype during which 50% of the cells down-regulate TrkA expression and instead upregulate the GDNF receptor c-ret (Molliver and Snider, 1997; Molliver et al., 1997). Trk receptors are, however not only involved in the patterning and selection by survival of the neurons. When TrkA was replaced with TrkC it caused a switch of phenotypes including changes in marker expression and central as well as peripheral innervation patterns, showing that Trk signalling is involved also in sensory neuron subtype specification (Moqrich et al., 2004).

**Specification of nociceptors**

Nociceptors all express TrkA and depend on NGF during development. The two transcription factors Brn3a and Klf7 have both been shown to be necessary for the
induction of TrkA (Lei et al., 2005; Ma et al., 2003; McEvilly et al., 1996). Runx1, a member of the Runt domain transcription factor family is specifically expressed in the nociceptive TrkA positive population (Levanon et al., 2001). It is able to induce ectopic TrkA expression, when overexpressed in the chick (Marmigere et al., 2006). Furthermore, reducing the levels of endogenous RuntB (chick homologue of Runx1) by siRNA, lead to a decrease in TrkA expression and a premature death of neurons (Marmigere et al., 2006). Recent data however has raised some questions on the role of Runx1 inducing TrkA. Using a conditional knock out mouse, with a Wnt1-cre driven excision of exon 4 of the gene, Runx1 was shown not to have any effect on survival of the TrkA neurons (Chen et al., 2006b). However, another study on a different loss of function mutant for Runx1, showed what seemed to be an increased death of the TrkA+ population in sensory ganglia (Theriault et al., 2004). In the Wnt1-cre mouse, Runx1 was shown to be necessary for TrkA down-regulation during the switch from TrkA to c-ret mentioned above (Chen et al., 2006b). This switch leads to the specification of two major groups of nociceptive neurons; TrkA+, c-ret+, peptide poor small diameter nociceptive neuronal sub type that projecting to lamina II in the superficial dorsal horn of the spinal cord, and that can be identified by their binding of the isolectin B4 (IB4) from Griffonia simplicifolia (Molliver et al., 1997). Second, a TrkA+, c-ret−, IB4 population expressing high levels of neuropeptides (e.g. Substance P) and that projects preferentially to lamina I and the outer lamina II. The onset of binding of IB4 was unperturbed in the loxed animals. This finding together with our finding in paper IV that IB4 binding is first observed at E18.5, which is at least one day before the down regulation of TrkA, suggests that Runx1 might be necessary for the completion of the switch but not its initiation. Chen et al used a knock out construct eliminating exon 4 of Runx1 but there are several different isoforms reported (Zhang et al., 1997). It is thus possible, that the lack of negative effects, as suggested from the chick experiments by Marmigere et al, on TrkA expression in cells lacking exon 4, is due to exon 4 being necessary for the loss of TrkA during the switch but not its initial induction. The central projections of putative IB4 positive neurons are set up prior to the switch excluding the lack of TrkA expression in the role of directing the neurites too lamina II (Molliver and Snider, 1997). In the light of this it is intriguing that cells lacking Runx1 all project to lamina I, suggesting that not all effects of Runx1 are derived from manipulation of TrkA signalling (Chen et al., 2006b). Runx1 was also shown to be necessary for the expression of several ion channels specific for nociceptors including TRPA1 and TRPM8. Interestingly, only the expression of high levels of TRPV1 (a very specific nociceptive marker, see below) in 2.6% of the cells, and not the intermediate in 40%, was dependent on Runx1 expression. Since there are reports on thinly myelinated nociceptive neurons expressing higher levels of TRPV1, this could imply that only the expression of TRPV1 in Aδ-fibers is affected by the loss of Runx1 (Ma, 2002). A result at least indicating that the effects of Runx1 is not
exclusive to the TrkA positive population is the loss of TRPV2 receptor expression, previously shown to be expressed by thickly myelinated TrkC expressing neurons as well as Aδ-fibers (Chen et al., 2006b; Tamura et al., 2005).

**Specification of proprioceptors**

Proprioceptors are the fastest conducting, thickly myelinated neurons that innervate joints, tendons and muscle spindles and are responsible for body posture and our knowledge of limb position in space (Ernfors et al., 1994). They can be divided into three subclasses Ia (primary muscle spindle afferents), Ib (Golgi tendon organs) and II (secondary muscle spindles) that innervate different structures and project centrally to different locations within the spinal cord (Brown and Fyffe, 1978; Brown and Fyffe, 1979; Hoheisel et al., 1989). Since body posture regulation is dependent on very quick corrections, the primary central innervation target of Ia afferents coming from muscle spindles are the motor neurons in the ventral spinal cord creating a short circuit of feedback between effectors and sensors (Brown and Fyffe, 1978). Transcription factors of the ETS family (Pea3 and Er81) are expressed in both post mitotic proprioceptors and their corresponding motor neurons in the spinal cord, and are upregulated in response to peripheral target derived signals (Arber et al., 2000; Patel et al., 2003). They are involved in later stages of proprioceptive development regulating target innervation and arborization as well as central axonal projections, and are responsible for setting up the sensory-motor circuit (Arber et al., 2000; Lin et al., 1998; Livet et al., 2002). Wnt3 has been identified as a signal controlling the sprouting of central proprioceptive projections in the spinal cord (Krylova et al., 2002). Runx3, another member of the Runt family of transcription factors, is involved in the specification of proprioceptors and seems to act by down-regulating TrkB expression in the TrkB⁺/TrkC⁺ positive neurons during development. It is also involved in the survival of proprioceptors, and the regulation of their central innervation of (Inoue et al., 2002; Levanon et al., 2002). The difference in the latter process between subtypes of proprioceptors seem to be due to a differential level of Runx3 expression (Chen et al., 2006a).

**3.4.3 TRP-channels and function**

Neurons of all sizes, with all thicknesses of myelin are represented in the mechanosensitive population, while the response to chemical or temperature stimuli is unique for the unmyelinated and thinly myelinated fibers. Trp-channels has emerged as the principal ion channels determining the receptive properties of sensory neurons and are preferentially found on this latter group (Vastani, 2005). In paper IV, using quantitative rtPCR and ratiometric calcium imaging, we studied the distribution of three TRP-channels among developing sensory neurons (Fig. 5).
Dissociated DRG cells from different developmental stages were plated onto coverslips. The cells were allowed to adhere and then live stained for IB4 and CTB before Ca\(^{2+}\)-imaging with the stimuli; cold, menthol, cinnamaldehyde, and capsaicin. Live staining image was then overlaid onto the film and each cell was individually scored. DRGs from all stages were also collected for quantitative rtPCR.
The hot TRPV1 and chili fruits

The chili fruit or paprika has made a substantial contribution to science in general and Hungarian science in particular. The Hungarian scientist Dr. Szent Gyorgyi won the 1937 Nobel Prize in concerning work on paprika and Vitamin C but it is in another context I shall consider it. Namely, the contribution of the paprika or chili fruit in the discovery of capsaicin. It was the Hungarian scientist P.A. Bucholtz who in 1816 discovered that the pungent principle of peppers could be extracted from the macerated pods with organic solvents. In 1846, L. T. Thresh reported that the pungent principle could be extracted in a crystalline state, he also gave the compound its name (Thresh, 1846). It had long been used by Indians of the new world for its analgesic effects and this use had spread also in Europe in the second half of the 19th century as noted by the Hungarian botanist-turned-medical scientist Hangay in 1887 (Hangay, 1887). Capsaicin is a stable alkaloid seemingly unaffected by cold or heat, retaining its original potency despite time, cooking, or freezing. It has no flavor, color, or odor but still, it is one of the most pungent compounds known, detectable to the palate in less than one to a million dilutions (Szolcsanyi, 1977). Capsaicin has been studied for centuries for its effects in heat and pain in mammals but not until the ground breaking discovery of its receptor TRPV1 (originally named VR1) have we understood the molecular mechanism behind its actions (Caterina et al., 1997).

TRPV1 is temperature sensitive in the noxious range and actually does not respond to capsaicin per se but rather is sensitized by these stimuli leading to a response to ambient temperatures (Tominaga et al., 1998). In DRG cultures from TRPPV1 knock out mice neurons fail to respond to temperatures in the range 43-50°C, which is the range suggested to activate TRPV1 channels according to studies in heterologous expression systems (Caterina et al., 2000). In some neurons the heat responses required temperatures of above 50°C and were unaffected in the TRPV1 knock out, indicating that another mechanism involved the detection of extreme temperatures. The response of neurons to temperatures above 50°C has been attributed to the expression of TRPV2 in medium and large sized, TrkC expressing myelinated neurons (Caterina et al., 1999; Tamura et al., 2005). When it comes to expression of TRPV1 in different subtypes and during development of the DRG there is a lack of consensus in the field. Of the different techniques used for studying the expression of TRP-channels rtPCR and functional studies have proven the most sensitive compared to immunohistochemistry and in situ hybridization, which is important to keep in mind when comparing findings.

Using in situ hybridization and immunohistochemistry, TRPV1 had been reported to be present at E13 but not E11 or E12 (Funakoshi et al., 2006; Tamura et al., 2005). We found only extremely low levels of TRPV1 mRNA level at E11.5 in less than half of the embryos along with sporadic responses to capsaicin. At E12.5 these responses were more common along with robust finding of mRNA however still only 10% of adult levels. The next stage that we investigated was E14.5, when we
could see a peak of responses to capsaicin with more than 60% of the cells responding. This is in agreement with the finding from in situ studies where Funakoshi et al claim that “a majority” of the cells express TRPV1 at E13.5 (Funakoshi et al., 2006).

In the adult, TRPV1 was originally described (using in situ hybridization) to be expressed in small diameter C-fiber neurons (Caterina et al., 1997). This still holds true even if percentages have had to be revised due to improved sensitivity of detection. It is also accepted that TRPV1 expression is found exclusively in nociceptive neurons (Breese et al., 2005; Caterina et al., 2000; Funakoshi et al., 2006; Ma, 2002; Woodbury et al., 2004) but not whether it is C-fiber specific or not. It is my belief however that the existing data supporting the TRPV1 expression within thinly myelinated Aδ-fibers is convincing (Kobayashi et al., 2005; Ma, 2002; Ringkamp et al., 2001; Vastani, 2005). With regards to the expression within the different subtypes there has been some controversy as to how much of the non-peptidergic IB4 positive neurons express TRPV1. In a study in mouse on GDNF and innervation only 2% of the IB4 population in lumbar L4 or L5 ganglia were shown to be immunoreactive for TRPV1 (using an antibody raised against rat TRPV1) (Zwick et al., 2002). In a following study also using immunohistochemistry but this time with an antibody against mouse TRPV1, this percentage was determined to 5.4-1.8 % depending on mouse strain used (Woodbury et al., 2004). This is in stark contrast to the functional finding where a much higher percentage of the IB4 neurons in L 4/5 ganglia were found reactive to capsaicin (Breese et al., 2005). The percentage in latter report (26%) is still lower than what we found in paper IV, where we show that 40% of the IB4 positive neurons respond to capsaicin using the same mouse strain as Breese et al. This high percentage seems to be set already early and exist throughout development. The difference between our paper and Breese et al is likely to be due to number of cells analysed, their study is based on patch clamp recordings, while extremely sensitive also cumbersome and therefore difficult to use for quantifications (Breese et al., 2005). Our numbers in the adult are based on the recording and staining of more than a thousand cells from several different animals increasing the accuracy. Another functional study using patch clamp techniques confirm our finding of high percentage of TRPV1 expression within the IB4’ population (Liu et al., 2004).

The cool TRPM8 and menthol
The cooling agent menthol has long been used in the study of cold perception (Hensel and Zotterman, 1951). Two groups, using different strategies, independently identified and cloned its receptor, TRPM8 (McKemy et al., 2002; Peier et al., 2002). They showed that TRPM8 is activated by innocuous cool stimuli in heterologous expression systems. Based on psychophysical evidence the perception of the innocuous cool sensation seems to be mediated by Aδ-fibers (Fowler et al., 1988; Fruhstorfer, 1984), while the excitation of C-fibers does not seem to evoke a conscious perception (Campero et al., 2001). TRPM8 was isolated due
to its response to menthol and several other stimuli (e.g. icillin, eucalyptus, and spearmint oil) has since been shown to activate the receptor but none are as specific as menthol (Bandell et al., 2004; Chuang et al., 2004; McKemy et al., 2002). It was originally shown to be expressed by 5-10% of DRG cells preferably in non-peptidergic non-IB4 small diameter neurons (McKemy et al., 2002) and this has been confirmed in other studies along with the expected expression in both TrkA positive C-fibers and Aδ-fibers (Kobayashi et al., 2005). This is in accordance to what we see in paper IV where we have around 7% of total neurons responding to menthol and those neurons which are mostly found in the IB4 negative population. There are data based on in situ suggesting a complete lack of overlap between TRPM8 and TRPV1 in studies on mouse and rat (Kobayashi et al., 2005; Peier et al., 2002). This is clearly not in accordance to our results in which we have a 44.7% percent of the menthol responsive cells also responding to capsaicin. This could however be due to limited sensitivity of the in situ technique. We identified neurons responding to cold stimuli but not to menthol within the IB4 positive population indicating the presence of a non-TRPM8, IB4 positive cold sensitive population. Previous reports on the development of TRPM8 had shown that its expression was detected by in situ at E16.5 or E17.5 (Chen et al., 2006b; Tamura et al., 2005), a finding confirmed in our paper IV, in which we see the first TRPM8 mRNA and functional responses at E16.5 with an increase to adult levels at E18.5.

The mysterious TRPA1

In a database search for relatives to known TRP-channels, an already cloned protein was found (ANKTM1), this was a channel that responded to noxious cold stimuli and icillin, but not menthol or capsaicin, when expressed in heterologous systems (Chinese hamster ovary cells; CHO) (Story et al., 2003) and was later renamed TRPA1 (Corey, 2003). In later studies, several pungent compounds, including mustard oil, cannabinoids, gingerol, and cinnamon-aldehyde, of which the latter seems to the most specific, were identified as agonists for this channel (Bandell et al., 2004; Jordt et al., 2004). They also raised serious doubt as to the role of TRPA1 in cold transduction in neurons by showing that only 4% of trigeminal neurons responding to mustard oil showed a cold response. This in contrast to the 36% of the cinnamonaldehyde responsive cells also responding to cold that we find in the DRG, but our data further supports the notion that TRPA1 does not convey cold in DRG neurons. Also questioned by our study is the reports claiming that all TRPA1 expressing cell coexpressed TRPV1 (Kobayashi et al., 2005; Story et al., 2003). We clearly see a cinnamon aldehyde responsive population that does not respond to capsaicin. This could once again be due to differences in sensitivity, a theory supported by the fact that we detect 9.6% responding to cinnamonaldehyde as compared to 3.6% by Story et al. This further stresses the importance of functional studies in this kind of studies. TRPA1 has a role in mechano transduction in the hair cells of the cochlea but its role in the DRG remains a unknown (Corey, 2003).
4 ISOLATION OF bNCSCs

4.1 bNCSC CULTURE

Here follows a detailed instruction on the dissection and culturing of the bNCSCs:

1. Dissect DRGs (Fig. 6) in cold N2 media (see below)
2. Collect ganglia in N2 media and keep on ice until dissociation
3. When all DRGs have been dissected (I usually do 7-8 embryos at a time) let them settle without centrifugation before removing supernatant
4. Add Collagenase/Dispase (1 mg/ml, Roche) and DNase (0.5 mg/ml, Sigma) into N2.
5. Incubate ganglia in above solution 20-30 minutes at 37°C. Shake once after half time.
6. After incubation allow ganglia to settle and then remove supernatant.
7. Rinse once in 500 μl N2 supplemented with B27 (1:50, Gibco) and bFGF and EGF (20 ng/ml, RnD Systems). Allow ganglia to settle before removing supernatant.
8. Add 500 μl new media (as above, B27, EGF and bFGF) and triturate carefully with fire polished Pasteur pipettes. The ganglia should fall apart quite easily.
9. Count cells and plate into untreated cell culture 24 well plates. Divide the cell suspension into desired number of wells (I usually use 3-4 wells for 7-8 embryos but aim for ~100,000 cells per well). Add up until 400-500 μl media per well.
10. Allow to settle for ~12 hours and then carefully exchange nearly all media in wells removing most of the non-adherent cells. Stem cells are adherent at this time point. I usually use 500 μl media per well in 24 well plates.
11. Change 50% of media every 2nd day or more every 3rd day.

Critical points:

Cell density at plating: try different densities and follow culture to find the optimal. There will be spheres/clumps forming early (maybe from rapidly dividing precursors). These later die or differentiate. Usually takes around 10 days for the real spheres to have grown at which time point most other cell types are gone.

For differentiation:

Coat plastic with PDL (50 μg/ml in water) at 37°C overnight, rinse properly before coating with Laminin (20 mg/ml in N2) for at least 3 h at 37°C but preferably overnight before changing medium and adding cells or spheres. Use N2-medium with B27 and NT3, GDNF, NGF, BDNF (all 10 μg/ml) and RA 100 nM.

N2-medium:

Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (D-MEM/F-12) (1X), liquid, 1:1, with L-glutamine and HEPES buffer with the addition of N2 supplement (1:100; Gibco)
Figure 6. Dissection of DRGs with adjoining boundary caps

Step 1) With the embryo pinned down on its back, cut with forceps along the entire length of the abdomen and remove all organs and intestines, including the carotid arteries (in red), baring the spinal column.

Step 2) If the embryo is E11.5 there is a “thread” running through the ventral vertebrae (in red), grab this at the rostral end and pull it out. This will create a groove into the vertebrae. Deepen this groove by gently running the forceps along the spinal column until the spinal cord is visible. If the “thread” is missing, you probably have an embryo of another stage, just carefully cut the middle of the vertebrae, making a similar groove, along the whole axis.

Step 3) Insert the forceps at 45° next to the spinal cord through the body wall and in that manner cutting a hole along the entire axis. Remove the lateral parts (the now loose body walls) from the head and rump, to reach the last figure.

Step 4) Grab the remaining tissue in the neck, beneath the spinal cord, and carefully pull it caudally revealing the DRGs. This step is increasingly easier the older the embryo. In younger stages (around E11) subsequent cleaning might be necessary removing bits and pieces of tissue remaining between and dorsally to the DRGs.

Step 5) Using L-shaped tungsten needles, cut the DRGs from the spinal cord by pressing the needles against the bottom of the dish. Be careful to avoid any spinal cord contaminations. At this step it also possible to detach the roots individually from the spinal cord and thus micro-dissect them (and the boundary cap) independently from the ganglia.
5 CONCLUSIONS

In this thesis I show that the boundary cap contains neural crest stem cells and that these bNCSCs have the ability to differentiate into multiple sensory neuron subtypes, including peripheral glia, and smooth muscle cells. The derived sensory neurons respond to typical sensory input and the Schwann cells can myelinate axons. Furthermore I have investigated the regulation of the stem cells during development and provided evidence suggesting that the boundary cap cells are regulated by GABA signalling, affecting their proliferation negatively. In the last part of the thesis I have identified the emergence of functional sensory neuron subtypes during development of the dorsal root ganglia.
6 ACKNOWLEDGEMENTS

I have had the pleasure to meet several people during this work and there are many to whom I owe gratitude. With my sentimental streak the list grows long, but please bear with me. Except for my dear brother Karl-Johan Hjerling, who helped me during long nights and on who I can blame the appearance of this thesis, and Patrik, Clare, Michael, Martin and Jorge for proof-reading, I also in particular would like to thank:


Malin: Tack för tiden vi fick tillsammans. All omtanke och kärlek kommer alltid att finnas kvar.

Professor Patrik Ernfors
Teacher, your school is a hard one. Your burning intensity, brilliant intellect and amazing ability to find the focal point of any question is astonishing. Thank you for pushing me forward, investing the trust in me to explore paths of my own, and for teaching me the importance of conveying science. I will long remember such adventures together as volcano climbing, Italian mid-town late night swimming, and unspoken skiing competitions. Thank you, for taking me under your wings but most of all for our friendship.

Professor Martin Koltzenburg
Your passion for both theoretical and applied science has been my inspiration on many a late night. Your admirable ability to lend confidence and your attention to detail still amazes me. Thank you for your incredible hospitality at work, and for introducing me to the (a) world of pain, also for many great nights in pubs and restaurants of London, good cigars, naff Swiss festivals, and for being a friend and mentor.

Collaborators across the world and campus:
Karolinska Institutet, Huddinge, Sweden: Mohammed Abdul H Göteborg University, Sweden: Professor Sven Enerbäck, Anna Cederberg, Mikael Heglind Umeå University, Sweden: Professor Thomas Edlund Austral University, Brazil: Professor Marcelo Villar Ecole Normale Supérieure, France: Professor Patrick Charnay, Piotr Topilko and Sophie Halliez. Thank you all for fruitful collaborations, and may future ones be as successful!
Also I would like to thank Professor Yves-Alain Bardes, University of Basel, Switzerland, for your generosity in welcoming me to the lab to learn techniques and for good discussions. Nicolas Plachta: for brilliant technique, an inquisitive mind and for showing me around Basel and parts of France.

The Ernfors lab, past and present:

Anna Stenqvist: My proud roommate, without your trust and support this would not have been. I have learnt many things from your honesty and would be a lesser man without our friendship. Your observations on human nature are second to none. Thank you for endless analytical discussions, warm hugs, and silly jokes. Karin Agerman: The original resmango and former roommate; your integrity, kindness, and hardheadness pulled me through the first years and you made a friend for life. You have taught me many things and I enjoy our discussions whenever we meet up. Michael Andäng: may the constant bursts of ideas, ranging from crazy to brilliant, never cease. Your kindness and generosity is an inspiration and I look forward to many future mind opening discussions and collaborations. Till barrikaderna! Jorge Aquino: a master in the art of photography and a sorcerer in histology. Thank you for glimpses of another way of life, your sincere concern, and for great collaborations. Frédéric Marmigère: for sharing the hard times and for useful lessons on human nature and task prioritization. Good luck in France! Miwako Kobayashi: for strange cookies, insights into Japanese culture and a fruitful collaboration. Susanne Elg: Two minds one thought, for all the fun and your honesty and straight forwardness. Good luck in suburbia! Andreas Montelius: you are an inspiration with your fascination for big biology. Also for always sharing your breakfasts. Dmitry Usoskin: for your unpretentious presentations, your straightforward attitude and that great bottle of vodka to accompany the delicious caviar. Kalle Lundberg: a true original. For your lack of respect, your drive, your impeccable sense for fashion and providing us with millions of stories. Let’s hope for future collaborations. Bengt Fundin and Tibor Harkany: for answering stupid questions and for passing on the art of microscopy to the next generation. Christel Baudet: for answering all those questions and for taking care of the lab. Paul Berghuis: your carefree attitude is an example to all of us. Esther Pozas: For your straightforward attitude. Orsolya Penz: for technical help and a sarcastic sense of humour. François Lallemend: for your incredible interest in the developing ear and for bringing a bit of silence to the office. Annika Ahlsén, Lotta Skoog, Claudia Tello-Lagos, Lena Amaloo, Jonny Söderlund and Annika Käller: for all the hundred small and big things. Also Guilherme Lucas, Sten Linnarsson, Åsa Mikael Edman, Philippe Naveilhan, Richard Warfinge, Anna Hjelmqvist, Hessameh Hassani, Alain Camillieri, Marton Dobsay, and all people passing through during my time in the lab for making it into the place it is.

The other labs at Molecular Neurobiology:
Professor Ernest Arenas: for the creative atmosphere, recruiting great people and for showing an interest in my project. Your initial guidance in the field of stem cells was invaluable. Professor Carlos Ibanez: for creating an inspiring atmosphere with your scientific clarity, your scary questions, and your support. Gonçalo Castelo-Branco: for a friendship unexpected but nevertheless true. For sharing Portugal, road trips, music sessions (why no one complained on your music remains a mystery, bastards!) and science at its best. Linda Edman: For dark and silent early mornings on frozen lakes (with or without skates, but always with hot coffee) and for running; the taste of blood in my mouth, while you run seemingly unaffected a few paces ahead. For your glittering laughter and sparkling persona always ready to lend a supportive hand. Good luck with the cxxrcs and maybe our paths cross soon. Nina Rawal: for those great loud discussions that has other people running for shelter, the lessons in self confidence, the scientific name dropping (you are still unbeaten) and for the advice on all difficult choices nearing the end. 6 weeks… Let’s hope we meet up in NYC! Olle Andersson: for the discussions on life in general and science in particular. For the great skiing and plenty of beer both in London and Stockholm. Keep up your fantastic attitude to science! Anita Hall: for remembering all those occasions whether difficult or happy. You are a generous friend and a scientific role model. Thanks for teaching me gardening in cell culture and for nights out. Clare Parish: for skiing and death defying pulkaåkning and pushing each other further, never backing down. Our discussions on life and science will stay with me. Adrian Brunkhorst: for never giving up, no matter what, our friendship and your dramatic streak. Pontus Holm: for your fantastic attitude towards academia and science and for football and music. Kyle Sousa: for your loud, incredible sense of humour and for always having good answers Joseph Wagner and Xavier Rodriguez: The old timers, thanks guys for all the help on setting up the stem cell cultures. And thanks again Xavier for getting me onto that shuttle going home from Keystone. Vita and Lenka Bryja: the slivovice and the damage done. Thanks for all the fun and for having me and Clare. Julianna Kele: for all your concern and all those late nights. Emma Andersson: for your smile and the amazing ability to get things done. Paola Sacchetti: for dragging me out in the forests and for driving me around in style Gunnar Schulte: for keeping your priorities straight and your suavity. Good luck in your new lab! Anna-Lena Moliner: for taking care of my cells when I was stuck in England. Carmen Salto and Lottie Jansson-Sjöstrand: for keeping our lab in order. And all other past or present members of the lab keeping the banner of molecular neurobiology flying high.

The Neural Plasticity Unit at ICH, UCL, London:
Mona AlQatari: for generosity, precision work, tidiness, and amazing shoes. Konrad Maurer: My very Swiss friend, for the fascination over optics and for your enviable outlook on life. Clare Munns: Keep up the good work and you healthy
scepticism! **David Barker**: for your enthusiasm and great sandwiches. Good luck with those waves.

**Max Delbrück Centre, Germany, past and present:**

**Professor Paul Heppenstall**: England! Your friendship alone made the whole trip worthwhile. **Professor Frank Pfrieger**: for your encouraging words, starting me off in science and for still being a role model. **Jung-Bum Shin**: thanks for all the Kimchi, car rides and for taking me to the Jung-Bum bar. **Professor Gary Lewin**: for referring me to Patrik. He still believes that I worked in your lab. And all the others that made my time memorable

All other friends in science: no one mentioned, no one forgotten…

**Martin Rieman, Peder Jogstad, Ingrid Frykman, and Professor Martin Kanje**: för att ni givit mig inspiration, kunskap och uthållighet, men framför allt för er förmåga att dela med er av er själva.

To my friends outside KI, the incredibly patient people still in contact in spite of me never having the time:

To my die hard unruly friends from the undergraduate studies in Lund: Dr. Marcus Stensmyr, Dr. Andreas Larsson, Dr. Johan Bylund and Dr. Nils Wierup (or **Mekon, Skägget, Jofa and Nice**, if you prefer): Last in line; our deed is done. So be it gentlemen, so be it…

**Carl**: Min gamle vän, tack för att du hjälper mig att se saker ur, om inte nyktert, så i alla fall ett annat perpektiv och för att du aldrig slutar fråga om jag ska med ut. **Malin and Ian**: for having me all those times in London and Stockholm, introducing me to Annika and the fantastic dinners keeping me alive during the late phase of writing. **Christian**: för din inställning till livet, många roliga mil, Öland och Indigo. **Mirja**: för din ärlighet och ditt härliga smittande skratt. **Magnus**: din enorma generositet, ditt snille och många sena diskussioner. **Eva and Neville**: for taking care of a weary Swede, your grand hospitality and the invigorating Sunday lunch discussions. **Stine, Aisa, and Jo**: for the small gestures, your patience and for all that time Annika occupies the phone. **Mark and Maria**: för att ni alltid hör av er, för segling, middagar och fester. **Anna**: som startade resan tillsammans med mig. **Kalle på Riche**: för känslan av att alltid vara välkommen. And all other friends that helped me keeping in touch with the outside world…

**Släkt** i norr o söder: Tack för ert stöd och uppmuntran.
Mina familjer,
Till er alla: tack för all hjälp inför disputationen!

Kjellströms o Plahns (Barbro, Per, Åsa och Magnus): tack för att ni så varmt har tagit emot mig i er familj, för söndagsmiddagar och lata dagar på Högholmen.

Wilhelmssons (Anette, Puffen och David): för allt stöd och varma samtal om trams och allvar och för strävan att bygga något nytt.

Barbro: för din lojalitet och för nya infallsvinklar på livet genom alla samtal genom åren.

Min fantastiska syskon Åsa, Daniel och Lizzie: tack för att ni alltid finns där!

Kalle: bror och bästa vän. Utan dig att hålla i när det blåser skulle jag vara förlorad.


Och till Annika, min älskade. För vår framtid!
7 REFERENCES


