Genetic control of sensory neuron diversification

Marina C M Franck
From the Division of Molecular Neurobiology
Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

The somatosensory system of vertebrates transmits information from external and internal environments to the brain. This information relates to various modalities such as touch, temperature, itch and pain. The different modalities require a variety of subtypes of sensory neurons, tuned to detect and transmit specific stimuli. Each of these subtypes expresses a specific set of proteins to serve this highly specialized function and to control the cell type specific gene expression. This thesis explores the development and diversity of sensory neuronal subtypes in the dorsal root ganglion (DRG) of the mouse.

In the five studies included in this thesis, we have investigated the roles of several genes in the development and function of sensory neurons. In Paper I, the focus is on a transcription factor, Cux2. We described that its expression is limited to large, early born neurons, which are mainly mechanosensitive, including a lineage of poorly characterized large TrkA+ neurons. We found no evidence that Cux2 would affect neuronal subtype specification, but instead we showed that it contributes to regulation of mechanosensation.

Transcription factors themselves are closely regulated in order to be expressed at the right time and place in development. In Paper II we identified that FGF signaling from earlier-born neurons triggers the upregulation of the transcription factor Runx1 early in the development of the thermo-nociceptive lineage. Signaling by soluble factors is also involved in the late stages of maturation of neuronal identity, as we demonstrated in Paper IV for the Ret receptor. We reported that the loss of Ret expression caused a hypersensitivity to several sensory modalities and showed that Ret is necessary for the expression of a large number of ion channels and receptors. One of the Ret-regulated genes was the cold receptor TrpM8. In Paper III we showed that TrpM8 expression was confined to a small population of neurons lacking coexpression with most subtype markers. We also characterized the developmental expression of all members of the TrpM family in the DRG and showed that most of them were expressed with individual temporal patterns.

Finally, in Paper V, we characterized the expression pattern of the enzyme Tyrosine hydroxylase (TH), the function of which is unknown in the DRG. TH is central in the catecholamine synthesis pathway, but whether or not that pathway is active in the DRG is uncertain. We showed that neurons expressing TH belong to the Ret+ population and that the expression of TH depends on Runx1 but not Ret.

In summary, we have described a number of novel sensory neuron populations as well as genetic mechanisms governing development and diversification of specific populations. These results lead to a better understanding of the somatosensory system and hopefully in extension to better treatments for patients with somatosensory disturbances such as chronic pain conditions.
LIST OF PUBLICATIONS

I. Isabelle Bachy, Marina C M Franck, Lili Li, Hind Abdo, Alexandre Pattyn, Patrik Ernfors
   The transcription factor Cux2 marks development of an A-delta sublineage of TrkA sensory neurons
   Developmental Biology (2011) 360:77–86

II. Saïda Hadjab-Lallemand*, Marina C M Franck*, Yiqiao Wang, Ulrich Sterzenbach, Patrik Ernfors, Francois Lallemand
    Induction of Runx1 in TrkA+ sensory neurons by a local source of FGFs
    Manuscript

III. Susanne Staaf, Marina C M Franck, Frédéric Marmigère, Jan P Mattsson, Patrik Ernfors
     Dynamic expression of the TRPM subgroup of ion channels in developing mouse sensory neurons

IV. Marina C M Franck, Anna Stenqvist, Lili Li, Jingxia Hao, Dmitry Usoskin, Xiaojun Xu, Zsuzsanna Wiesenfeld-Hallin, Patrik Ernfors
    Essential role of Ret for defining non-peptidergic nociceptor phenotypes and functions in the adult Mouse
    European Journal of Neuroscience (2011) 33:1385–1400

V. Marina C M Franck, Moritz Lübke, Jaime R James, Patrik Ernfors.
   Characterization of TH-expressing sensory neurons during mouse development
   Manuscript

* equal contribution
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>C-LTMR</td>
<td>C-low threshold mechanoreceptor</td>
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<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>DRG</td>
<td>dorsal root ganglion</td>
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<td>E</td>
<td>embryonic day</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>eRet</td>
<td>early Ret$^+$</td>
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<tr>
<td>eTrkA</td>
<td>early TrkA$^+$</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>Fgfr1</td>
<td>Fibroblast growth factor receptor 1</td>
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<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<td>GFL</td>
<td>GDNF-family ligand</td>
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<td>GFRα</td>
<td>GDNF family receptor alpha</td>
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<td>isolectin B4</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>iTrkA</td>
<td>intermediate TrkA$^+$</td>
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<td>I Ret</td>
<td>late Ret$^+$</td>
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<tr>
<td>lTrkA</td>
<td>late TrkA$^+$</td>
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<tr>
<td>Mrgrp</td>
<td>Mas-related G protein-coupled receptor</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament 200 kDa</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>Neurogenin</td>
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<td>Neurotrophin-3</td>
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<td>NT4</td>
<td>Neurotrophin-4</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
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<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>Ret</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
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<td>Trp</td>
<td>Transient receptor potential</td>
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1 INTRODUCTION

In order to interact with the environment, organisms need to obtain information both about the environment and about their own bodies. Sensation of both physical (e.g., movements, gravity, temperature, electromagnetic fields and waves) and chemical (molecules dissolved in the surrounding medium or present in the organism itself) cues is important. The five classical senses of humans (vision, hearing, taste, smell and touch) were described already by Aristotle (Johansen, 2007). Later, additional senses have been added such as the senses of balance, temperature, pain and itch. In mammals the somatosensory system detects a wide variety of both physical and chemical stimuli in and on the surface of the body. In order to do this, animals rely on specialized sensory neurons, which are equipped with specific receptors, and in some cases end organs, to amplify and transform relevant sensory stimuli into nerve impulses. As the nature of the stimuli is so diverse, so are the neurons that detect and transmit them.

In the end of the 19th century, Maximilian von Frey promoted the idea that different modalities of somatosensation could be attributed to specific anatomical structures. This built on the discoveries and theories of earlier research during the 19th century (reviewed by Norrsell et al., 1999). The Polish scientist Ludwig N. Natanson might have been the first to provide evidence for different types of sensors in the somatosensory system in his article from 1844 (Natanson, 1844). Based on observations of when a limb falls asleep, he proposed that the sense of touch could be composed of three different independent organs that were inactivated in a reproducible order and were responsible for sensations of temperature, pressure and touch/tickle. Further evidence for different sensory modalities was provided through the independent discovery of discrete cutaneous sensory spots by three scientists in the 1880's. These three; Magnus Blix at Uppsala University in Sweden, Alfred Goldscheider in Germany and Henry Donaldson at Johns Hopkins University in Maryland, USA, reported that stimulation of discrete spots on the skin would elicit different sensations of pressure, cold or warmth. However, the nature of the pain modality was disputed at this time. Maximilian von Frey united the notions that forceful stimuli could elicit pain at most locations and that some cutaneous points seemed more likely than others to elicit painful sensations. He proposed the existence of separate high-threshold and low-threshold detectors and that separate receptors mediate sensations of warm, cool, touch and pain (Norrsell et al., 1999).

The theoretical discussion about the nature of the somatosensory system has from the 19th century until today largely been disputing two opposing theories laid forward in the 19th century. The "law of specific energies" or "labeled line theory" was originally formulated for the five general senses in 1826 by Johannes Müller and later promoted by Maximilian von Frey for the somatosensory system. This theory suggests that each receptive unit is tuned for a specific kind of stimulus and regardless of how the unit becomes activated, the mind will interpret its signals as signals of that specific stimulus.
The opposing theory is the "population coding theory" or "pattern theory", which suggests that the nature or intensity of the stimulus or combinations of activities in different units are accountable for different sensations. The disputes in the 20th century particularly concerned the nature of pain sensations, since it was observed that pain can be elicited by most kinds of stimuli (mechanical, hot or cold) if only the intensity of the stimulus is high enough.

Although already von Frey suggested that free nerve endings would be responsible for pain sensations, and several lines of evidence during the 20th century pointed towards an involvement of the unmyelinated subset of peripheral neurons in pain sensation (Norrsell et al., 1999), it was not until 1969 that the existence of devoted nociceptors (receptors for pain) was demonstrated experimentally (Bessou and Perl, 1969). Bessou and Perl (1969) performed nerve recordings of individual C-fibers in anesthetized cats and demonstrated that a large number of these individual neurons would respond only to high-threshold stimuli, indicating that they provided the sought labeled line for pain. This discovery sparked studies of the specific nociceptors as targets for pain therapies.

The genetic revolution in the late 20th century lead to a search for genes coding for functional proteins expressed in specific subsets of sensory neurons, that would explain their different functional properties. A remarkable milestone was the cloning of the capsaicin receptor TrpV1, which alone was sufficient to make cells responsive to heat and capsaicin (Caterina et al., 1997). The expression of this channel in a limited population of sensory neurons could explain the heat sensitivity of these cells, and thus elegantly demonstrate the basic mechanism for how different neuronal populations attain different modalities.

1.1 DEVELOPMENT OF SENSORY NEURONS

In vertebrates, somatosensory neurons are located in dorsal root ganglia (DRG), which are situated at each segmental level on both sides of the spinal cord and innervate the trunk and limbs, and in cranial ganglia innervating the head. The aforementioned ones of the mouse are the focus for this thesis, and unless otherwise stated, the conditions described are those that apply to the mouse (Mus musculus). These sensory neurons develop from a common precursor cell type in the neural crest, which undergoes several lineage splits over development and forms functionally different subpopulations of sensory neurons. The first and most fundamental split is the one between myelinated and unmyelinated neurons. The myelinated neurons have larger cell bodies and a faster speed of transmission than unmyelinated ones. They mediate various kinds of mechanosensation: touch sensations from the skin and proprioceptive information from muscles and tendons. The unmyelinated neurons mainly sense temperature and pain and are of a smaller size. Myelination correlates with the expression of different neurofilament proteins which can be used as markers for the different subtypes. Neurofilament 200 kDa (NF200) is preferentially expressed in myelinated neurons and Peripherin in unmyelinated ones (Ferri et al., 1990; Troy et al., 1990; Lawson and
There is a small population of medium-sized neurons that express both neurofilaments, and that are generally believed to be lightly myelinated neurons with Aδ fibers (Ferri et al., 1990; Lawson:1991ti, see also Ruscheweyh et al., 2007).

The neurotrophic tyrosine kinase receptors Tropomyosin-related kinase (Trk)A, TrkB and TrkC (mouse genes Ntrk1, Ntrk2 and Ntrk3 respectively) and the Ret receptor are crucial for survival and specification of different subsets of sensory neurons and also have been used as valuable markers to identify these subsets (Mu et al., 1993; McMahon et al., 1994; Molliver et al., 1997). TrkA and Ret are expressed in small- and medium-size neurons as well as small specific subsets of large myelinated neurons, while TrkB and TrkC are expressed in distinct subsets of medium to large size myelinated neurons (reviewed by Lallemend and Ernfors, 2012).

1.1.1 From neural crest to committed neurons

Sensory neurons of the DRG originate from the neural crest, a transient tissue formed at the edges of the folding neural plate. At the time of neural tube closure, neural crest cells undergo epithelial-mesenchymal transition, delaminate and migrate out on each side of the neural tube to various locations in the body (His, 1868; Weston, 1963). Future sensory neurons and glia coalesce to form a pair of DRG at each axial level. The migrating neural crest cells are multipotent and able to form a variety of structures depending on cues from the local environment (Le Douarin et al., 1977).

The neural crest cells giving rise to the sensory neurons become committed to a neuronal phenotype and exit the cell cycle during or after migration from the neural tube, around embryonic day (E)9.5 to E10.5 in mouse (Marmigère and Ernfors, 2007). The first cells to exit the cell cycle become large myelinated neurons, while later-born neurons predominantly become small unmyelinated neurons (Lawson and Biscoe, 1979). Neurogenesis of these two general classes of neurons corresponds to the timing of expression of two neurogenic transcription factors of the bHLH family, Neurogenin (Ngn)2 and Ngn1 respectively (encoded by the mouse genes Neurog2 and Neurog1, Ma et al., 1996; 1999). However, genetic tracing using Ngn2CreERT mice has shown that Ngn2+/ cells give rise to both glia and neurons of all classes without any preference for myelinated neurons (Zirlinger et al., 2002). Furthermore, in the absence of Ngn2, Ngn1+ cells can give rise to all neuronal subtypes (Ma et al., 1999). Therefore it is likely that Neurogenins are not involved in specification of neuronal subpopulations. They are, however, necessary for the formation of sensory neurons, since double knockout mice lacking both Ngn1 and Ngn2 do not form any DRG at all. The putative effects on neurogenesis are difficult to separate from the apoptotic cell death that happens early in both Ngn2 and Ngn1 mutant mice, indicating that Neurogenins are directly or indirectly required for survival (Ma et al., 1999). In other words, the apoptotic cell death may either be caused by absence of neurogenesis or be induced before the cells reach the onset of neurogenesis.
A later group of sensory neurons derive from the boundary cap, a transient structure of cells at the dorsal root entry zone at the surface of the neural tube. This population expresses both Ngn1 and Ngn2 and migrates to reach the DRG starting around E11.5. Most of the cells from the boundary cap become small TrkA+ neurons (Maro et al., 2004; Hjerling-Leffler et al., 2005).

A number of transcription factors have been identified acting in the process of neurogenesis and early diversification (Figure 1). Sox10 is expressed in neuronal and glial precursors and is necessary to maintain multipotency, but is rapidly downregulated upon neurogenesis (Kim et al., 2003). Brn3a (gene name Pou4f1) is expressed in some Sox10+ cells but is also maintained after Sox10 downregulation and regulates the transition from neurogenesis to terminal differentiation (Montelius et al., 2007; Lanier et al., 2009). After downregulation of Sox10, cells quickly start expressing early neuronal markers Foxs1 and Isl1. Isl1 acts together with Brn3a to drive differentiation into functional subtypes (Dykes et al., 2011). Remarkably, although Isl1/Brn3a compound knockouts do undergo neurogenesis, they have a severe diversification-defective phenotype lacking expression of any subtype specific markers including Trk receptors, Ret, Runx1 and Runx3 (Dykes et al., 2011). It is possible that Isl1 and Brn3a partially exert specification functions on separate sets of sensory neurons. Loss of Brn3a tends to more severely affect markers for early myelinated neurons such as TrkC, Runx3 and ER81 (gene name Etv1), while Isl1 has an important role for survival and maturation of unmyelinated neurons past E12.5 and a lesser role for myelinated neurons (Sun et al., 2008; Dykes et al., 2011). The role of Foxs1 is less clear, since its deletion does not cause any detectable disturbances of sensory neuronal phenotypes (Heglind et al., 2005).

**Figure 1.** Approximate timing of transcription factor expression in early DRG development.

*Based on data from Montelius et al. (2007)*
1.1.2 Trk and Ret signaling in DRG development

1.1.2.1 Trk signaling

Signaling by neurotrophin ligands through Trk receptors is key for survival and differentiation of specific subpopulations of sensory neurons. TrkA is the receptor for nerve growth factor (NGF), TrkB for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4; in the mouse encoded by the gene Ntf5) and TrkC for neurotrophin-3 (NT3; in the mouse encoded by the gene Ntf3). Trk receptors are receptor tyrosine kinases that upon ligand binding dimerize, autophosphorylate and activate intracellular signaling cascades (Greene and Kaplan, 1995). The common neurotrophin receptor p75 (in the mouse encoded by the gene Ngfr), which belongs to the tumor necrosis factor receptor family, also binds neurotrophins and modulates their signaling by forming complexes with Trk receptors. p75 can also signal independently of Trk receptors, often in complex with other receptors and mediating pro-apoptotic signals (Rabizadeh et al., 1993; Simi and Ibáñez, 2010; Ibáñez and Simi, 2012).

Data from embryonic stainings, knockouts and lineage tracing studies, have shown that both TrkC and TrkA are broadly expressed in early sensory ganglia (Ernfors et al., 1992; Smeyne et al., 1994; Fünfschilling et al., 2004). At E11.5 TrkC is expressed in most neurons and its expression overlaps largely with TrkA expression, but at E12.5 the number of TrkC+ cells is decreased. This coincides with an increase in cell numbers in the DRG due to proliferation of late-born neurons (Fariñas et al., 1998; Sun et al., 2008). A lineage tracing study using TrkC<sup>Cre</sup> mice crossed to Rosa26<sup>EGFP</sup>, that activate expression of enhanced green fluorescent protein (EGFP) cell-autonomously upon expression of TrkC, has indicated that approximately 70% of mature TrkB+ neurons and 20% of TrkA+ neurons have expressed TrkC at some point during their development (Fünfschilling et al., 2004). The latter ones likely correspond to the early TrkA population that we describe in Paper I. This population consists of early born neurons and covers approximately 20% of TrkA+ neurons at postnatal stages.

TrkA is only expressed in a small subset of neurons at E11.5, likely corresponding to the early TrkA neurons. Broad expression of TrkA can be seen from E13.5 when most late-born neurons have formed. Knockouts of NGF or TrkA show a selective loss of small neurons, indicating a specific requirement for TrkA for their survival (Crowley et al., 1994; Smeyne et al., 1994). The early large TrkA neurons depend on TrkA signaling for survival and/or for expression of TrkA, indicated by a complete loss of TrkA expression in NGF<sup>−/−</sup> DRG (Crowley et al., 1994). A similar loop of TrkA signaling promoting the expression of TrkA has been identified in sympathetic neurons (Deppmann et al., 2008). NT3 and TrkC knockouts each show a loss of lumbar DRG neurons (55% loss in NT3 mutants, 19% loss in TrkC mutants), including a complete loss of proprioceptive afferents (Klein et al., 1994; Ernfors et al., 1994b). The different sizes of the affected populations are likely to reflect actions of NT3 on TrkC+ cells through signaling via other Trk receptors. TrkC has indeed been shown to be able to
activate other Trk receptors in DRG neurons \textit{in vitro} (Davies et al., 1995). For the TrkB pathway, both BDNF and TrkB knockout mice lose around 30 \% of lumbar DRG neurons (Klein et al., 1993; Jones et al., 1994; Ernfors et al., 1994a). The fact that the loss of neurons in knockouts of the different Trk signaling pathways adds up to more than 100 \%, indicates that some neurons depend on different neurotrophins at different time points.

Knockouts for the different Trk receptors and their ligands have proved valuable tools for the fundamental understanding of sensory neuron populations. However, the effects of Trk signaling on differentiation are often masked by massive apoptosis in knockouts. For this purpose, compound knockouts lacking both Trk receptors and the proapoptotic BCL2-associated X protein (Bax) have been developed. The Bax protein is a regulator of apoptosis in development and mice lacking functional Bax have an almost complete inhibition of apoptosis in the nervous system, as well during the period of natural cell death as upon withdrawal of trophic factors (Deckwerth et al., 1996; White et al., 1998). Using compound knockout mice lacking both Bax and a TrkA or TrkC receptor, the survival-promoting effects of Trk signaling have been uncoupled from other functions. This has demonstrated roles for Trk signaling in peripheral innervation and expression of functional molecules (Patel et al., 2000; 2003).

\subsection*{1.1.2.2 Ret signaling}

Ret (Rearranged during transfection) is a transmembrane receptor tyrosine kinase, initially identified as a proto-oncogene with roles in several cancer forms, including thyroid cancer and multiple endocrine neoplasia type 2A and 2B (Takahashi et al., 1985; Arighi et al., 2005). The Ret receptor is a tyrosine kinase receptor with the unusual characteristic that it cannot bind ligands directly but requires ligand binding by a GDNF family receptor alpha (GFR\(\alpha\)) coreceptor. The coreceptors are glycosyl phosphatidylinositol (GPI)-linked to the external cell membrane and lack transmembrane domains. There are four members of this family, GFR\(\alpha\)1-4, which bind with variable affinity to four GDNF-family ligands (GFL). Upon ligand binding the GFR\(\alpha\) coreceptors associate with Ret and activate Ret signaling. The main interaction pairs are outlined in Table 1.

Ret signaling is involved in several developmental processes, as indicated by the effects of genetic deletion of GFL, GFR\(\alpha\) or Ret in mice. In kidney development GDNF from the metanephric mesenchyme is required for the induction and branching of the ureteric bud, and absence of Ret or GDNF causes severe defects in kidney morphogenesis (Pachnis et al., 1993; Schuchardt et al., 1994; Moore et al., 1996). In the enteric nervous system, Ret signaling is required for migration of progenitor neurons and GDNF or Ret knockout mice completely lack the enteric nervous system (Schuchardt et al., 1994; Moore et al., 1996). GFR\(\alpha\) receptors can also be cleaved off and act as soluble receptors, which are able to bind to other cells \textit{in trans} (Paratcha et al., 2001).
Receptor | Ligand | References |
--- | --- | --- |
GFRα1 | Glial cell line-derived neurotrophic factor (GDNF) | Jing et al., 1996; Treanor et al., 1996 |
GFRα2 | Neurturin | Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997 |
GFRα3 | Artemin | Baloh et al., 1998; Naveilhan et al., 1998; Widenfalk et al., 1998 |
GFRα4 | Persephín | Enokido et al., 1998; Lindahl et al., 2001 |

**Table 1. The GFRα coreceptors and their cognate ligands.**

Ret signaling requires the association of Ret with one of the coreceptors with bound ligand.

The intracellular domain of Ret contains several signaling tyrosine residues that upon phosphorylation bind different linker proteins and activate different intracellular signaling pathways (de Groot et al., 2006). Three functional isoforms of Ret are expressed dependent on alternative splicing of the 3’ end of the transcript. These are called Ret9, Ret43 and Ret51, according to the number of additional amino acid residues after the last common residue (Tahira et al., 1990; Myers et al., 1995). Ret9 seems to be the most important isoform in vivo and Ret9 knock-in mice, which only express the Ret9 isoform, appear to develop without great disturbances, in contrast to Ret51 knock-in mice (de Graaff et al., 2001). However, a specific NGF-dependent and GFL/GFRα independent phosphorylation of Ret51, but not Ret9, has been identified in sympathetic neurons and this signaling was shown to promote cell growth and metabolism (Tsui-Pierchala et al., 2002). This indicates that Ret51 might serve specific functions that are independent of Ret9. The Ret43 isoform is the most recently discovered and least characterized (Myers et al., 1995).

GFRα1 can also associate with Neural cell adhesion molecule (NCAM), thereby forming a complex to which GDNF can bind and signal independently of Ret (Paratcha et al., 2003). This signaling pathway regulates cell migration, neuronal morphology and synapse formation. A recent study shows that the Drosophila GFRα homolog DmGfrl does not act as a coreceptor for the Drosophila Ret homolog DmRet, but instead binds the NCAM homolog FasII (Kallijärvi et al., 2012). This indicates that the GFRα-NCAM interaction might be evolutionarily older than the interaction between GFRα and Ret.

### 1.1.3 Diversification of sensory neurons

The diversification process allows the development of the many sensory neuronal subtypes of the DRG serving various functions. Diversification has come out as a hierarchical process in which cell lineages split up and diversify over time. However,
we often do not know exactly at which stages diversification and fate specification occur, as it might be earlier than our markers can discriminate. On the other hand, loss-of-function studies have shown the importance of specific genes for the emergence of certain lineages, and in combination with data about the timing of expression of these genes have allowed us to draw conclusions about the timing of lineage segregation events. A model of our current understanding of the diversification process is outlined in figure 2.

1.1.4 Diversification of myelinated sensory neurons

Although most large neurons express Ngn2 during neurogenesis, Ngn2 seems neither to specify the fate of these neurons nor to be a specific marker for them (Ma et al., 1999).

Figure 2. Lineage relationships of sensory neurons. 
Adapted from Lallemend and Ernfors (2012), reproduced with permission.
Instead, the earliest marker for the future large myelinated neurons is TrkC, followed by Runx3, and in some TrkC⁺ cells, TrkB and Shox2 (Phillips and Armanini, 1996; Fariñas et al., 1998; Levanon et al., 2001; Kramer et al., 2006; Abdo et al., 2011; Scott et al., 2011). Some transcription factors have been identified to play instructive roles for the expression of these genes. In the trigeminal ganglion, Brn3a is needed for initiation of Runx3 expression (Dykes et al., 2011). Regarding the regulation of TrkC, Isl1⁻/⁻ mice show a delayed onset of TrkC expression, while Runx3 is needed for TrkC expression at stages around E12.5 (Inoue et al., 2002; 2007; Nakamura et al., 2008; Sun et al., 2008). The latter effect could partly be attributed to an increased apoptosis in the absence of Runx3 at E12.5. However, in compound knockouts lacking Runx3 and Bax the intensity of TrkC labeling is also decreased (Lallemand et al., 2012). Runx3 is also required for expression of proprioceptive markers ER81 and Parvalbumin in TrkC⁺ neurons (Inoue et al., 2007; Nakamura et al., 2008; Lallemand et al., 2012).
TrkC expression re-emerges around E15.5 in Runx3 knockouts, possibly in a different subpopulation than the one expressing TrkC at early stages (Inoue et al., 2002; 2007; Nakamura et al., 2008). While proprioceptive TrkC+ neurons clearly depend on Runx3 at E10-11, skin mechanosensitive neurons that also express TrkC are less affected by Runx3 loss, indicating that these might upregulate Trk later in a Runx3-independent manner (Nakamura and Morrison, 2008).

A subset of TrkC+ neurons initiate TrkB expression around E11.5 and separate TrkB+ and TrkC+ populations develop from this early double positive population (Kramer et al., 2006). Knockout experiments have indicated an instructive role of NT4 for the development of the TrkB+ subset. In the absence of NT4, TrkB+ expression fails to appear and the number of TrkC+ neurons is increased, indicating that presumptive TrkB+ neurons adopt a TrkC+ fate instead (Liebl et al., 2000). The instructive effect of NT4 might be mediated by regulation of Runx3 and Shox2, which have been identified as key transcription factors for the diversification of the TrkB+/TrkC+ lineage. Runx3 promotes a TrkC+ fate and Shox2 a TrkB+ fate, as outlined in Figure 3. The role of Runx3 is achieved by suppressing Shox2 and TrkB and allowing the development of TrkC+ neurons. At a molecular level, a silencer has been found in intron 7 of the Ntrk2 gene (encoding TrkB), which contains Runx binding sites and requires a combination of Runx3 and TrkC signaling for repressor activity (Inoue et al., 2007).

Figure 3. Gene regulation in the diversification of the TrkB+ and TrkC+ lineages.

NT4 signaling via TrkB might regulate Runx3, which in turn suppresses expression of Shox2 and TrkB. If Runx3 is downregulated, Shox2 is derepressed and can promote expression of TrkB. Note that the regulation of Runx3 by NT4 signaling is entirely hypothetical (question mark).
downregulate Runx3, Shox2 represses TrkC and promotes a TrkB+ phenotype (Abdo et al., 2011). Shox2 does not affect Runx3 expression, and thus the cellular fate decision depends on Runx3 expression. Possibly NT4 signaling via TrkB in early TrkB+/TrkC+ neurons could have a suppressive effect on Runx3 expression, however, this has not been addressed experimentally. At later stages, cutaneous slowly adapting mechanoreceptors have been shown to require BDNF for their function, but not for survival (Carroll et al., 1998).

Loss of Shox2 causes a decrease in the number of TrkB+ neurons accompanied by deficits in some of their peripheral targets, including; decreased Merkel cell innervation in both glabrous skin and guard hair follicle touch domes, loss of Meissner corpuscles and smaller deficits in Pacinian corpuscles and guard hair lanceolate endings (Abdo et al., 2011). This demonstrates the importance of the interaction between the peripheral nerve and the sensory end organ for the formation of the latter. The spared TrkB+ cells in Shox2 mutants may correspond to Aδ fibers that would develop from a separate lineage, possibly the early or intermediate TrkA lineage (see chapters 1.1.4.2 and 1.1.5.1 below). Unlike many other transcription factors like Brn3a, Isl1 and Runx3 which are expressed postnatally, Shox2 is a purely developmental regulator in the DRG and its expression is very low or absent after birth (Abdo et al., 2011).

1.1.4.1 The Aδ population

Functional experiments have revealed two general subsets of medium-size neuronal populations with lightly myelinated Aδ fibers: D-hair mechanoreceptors mediate light touch and Aδ nociceptors mediate early, sharp pain, in contrast to the pain mediated by the more slowly conducting C-fibers that is dull and difficult to localize. Temperature and itch sensitivity has also been attributed to some Aδ fibers. While molecular markers for the entire Aδ population have not been found, expression of the calcium channel Cav3.2 (Cacna1h) has been shown to be limited to D-hair associated neurons (Shin et al., 2003). Aδ neurons, like other myelinated neurons, also express NF200 and a subset of them express calcitonin gene-related polypeptide (CGRP, transcribed from the Calcitonin locus, Calca) (Ruscheweyh et al., 2007). Some careful studies on Aδ fibers have been done on the vibrissal hair follicles in the mystacial pad in mouse, which are probably most similar to D-hair neurons found in the hairy skin of the body. Lineage tracing of TrkC neurons, as well as results from Trk knockouts, have shown that the Aδ neurons of the vibrissal hair follicles express and depend on both TrkC and TrkA at some points during their development, but not in the adult (Rice et al., 1998; Fünfschilling et al., 2004). This is consistent with studies of D-hair mechanoreceptors in the DRG, which have been shown to depend on NT3 for survival in early postnatal development and on NT4 at adult stages, presumably through TrkC and TrkB signaling respectively (Stucky et al., 1998; 2002). Furthermore, a recent study indicates that D-hair afferents in hair follicles of adult mice are TrkB+ (Li et al., 2011). However, the molecular nature of Aδ thermo- / noci- / pruriceptors is less known. A subset of Aδ neurons has been shown to express TrpV1, respond to capsaicin and have a low heat
threshold (Treede et al., 1995; Nagy and Rang, 1999), but it is unknown whether this subset covers all or a part of the non-D-hair Aδ neurons.

1.1.4.2 The early TrkA population

Early lineages of large TrkA+ and Ret+ neurons can be identified from the earliest stages of DRG development. In Paper I we showed that early NF200+/TrkA+ (for simplicity referred to as eTrkA) neurons can be found as early as E11.5 when they constitute 18% of all neurons. Their absolute numbers do not change much over development although the percentage decreases as the DRG becomes populated by more small TrkA+ neurons. Due to their expression of NF200 the eTrkA neurons are believed to be Aδ neurons.

1.1.4.3 The early Ret population

Early large Ret+ (eRet) neurons can be found as early as at E10.5 (Pachnis et al., 1993; Molliver et al., 1997; Kramer et al., 2006). These neurons express MafA, NF200, GFRα2 and Kcnq4 and function as rapidly adapting mechanoreceptors with Aβ fibers innervating Meissner corpuscles, Pacinian corpuscles and longitudinal lanceolate endings in guard hair and auchen hair follicles (Bourane et al., 2009; Luo et al., 2009; Heidenreich et al., 2012). At early developmental stages before E13, e-Maf is a specific marker for these neurons, while later it is also found in other populations. (Wende et al., 2012). The subset of eRet neurons innervating hair follicles also express Npy2r (Li et al., 2011), and Met expression has been found in some large Ret+ neurons (Gascon et al., 2010). Some controversy exists whether or not eRet neurons innervate Merkel cells in touch domes of guard hair follicles, although the relative loss of such innervation in Ret conditional knockouts speaks for a role of Ret (Bourane et al., 2009; Luo et al., 2009). At P0, TrkB and TrkC mark separate subpopulations of eRet neurons (Bourane et al., 2009). Similar to Ret+/TrkB+ neurons, Shox2 is also required for the development of Ret+/TrkB+ neurons. Loss of Shox2 results in decrease of TrkB+ and increase of TrkC+ neurons within the eRet population around birth (Abdo et al., 2011; Scott et al., 2011).

1.1.5 Diversification of unmyelinated sensory neurons

1.1.5.1 The intermediate TrkA population

Unmyelinated neurons exit cell cycle later than myelinated ones and undergo neurogenesis at the time of Ngn1 expression and are dependent on Ngn1 for their proper formation and survival (Ma et al., 1999). TrkA and Runx1 are early markers for the small-cell lineage, which I will refer to as the intermediate TrkA (iTrkA) lineage to
distinguish it from the eTrkA population, which is a separate population of neurons (see chapter 1.1.4.2), and the late TrkA population, which forms from a subset of the iTrkA population (see chapter 1.1.5.2). Expression of TrkA and Runx1 in the iTrkA population require presence of Ngn1, likely due to a failure of neurogenesis and survival of small neurons in the absence of Ngn1 (Ma et al., 1999; Kramer et al., 2006). Also Brn3a and Isl1 regulate expression of Runx1 and TrkA (Dykes et al., 2011). Furthermore, in the trigeminal ganglion it has been shown that TrkA expression is regulated by Klf7 which, together with Brn3a, binds to an enhancer in the TrkA promoter (Lei et al., 2006). In the absence of Klf7, TrkA levels are severely decreased at E11.5-E13.5 (Lei et al., 2005).

At the earliest stages TrkA and Runx1 are regulated independently of each other. TrkA signaling is not required for the induction of Runx1, although it is needed for maintenance of Runx1 expression after E14 (Luo et al., 2007). Loss of Runx1 also does not have major effects on TrkA expression although a small transient decrease of TrkA at E12.5 has been reported in Runx1−/− mice (Kobayashi et al., 2012). However, in embryonic chicken DRG, interfering with Runx1 DNA binding sites using a splice variant of the Runx1 protein lacking transactivation domains, abolished TrkA expression in affected neurons. In addition, forced ectopic expression of Runx1 in migratory chicken neural crest induced expression of TrkA (Marmigère et al., 2006). These results indicate that Runx1 is both necessary and sufficient for TrkA expression in this system. However, the regulation mechanisms of Runx1 and TrkA may differ between chicken and mice. Moreover, binding of the truncated Runx1 protein to DNA regulatory sites may block access for other transcription factors required for TrkA expression.

There are indications that Runx1 affects proliferation of early DRG neurons through effects on the cell cycle (Yoshikawa et al., 2007; Kobayashi et al., 2012). Indeed proliferation is still ongoing in the DRG at the onset of Runx1 expression at E12.5 (Lawson and Biscoe, 1979; Chen et al., 2006). However, Runx1 is not expressed in Ki67+ proliferating precursors (Yoshikawa et al., 2007) and we show in Paper II that its expression is confined to TrkA− neurons, which are postmitotic (Huang et al., 1999). Thus, this regulation is likely non-cell autonomic.

The effects of Runx1 on neuronal subtype specification in iTrkA neurons during the embryonic period have been difficult to study due to the masking of early effects by later more prominent ones. However, Runx1 expression in the prenatal period is required for the later specification of a subset of neurons which downregulate Runx1 expression perinatally (see chapter 1.1.5.4 below) (Samad et al., 2010). It is possible that early effects of Runx1 are likewise required in neurons that additionally depend on continuous Runx1 expression.
A subset of unmyelinated neurons change from TrkA to Ret expression over the period from E16.5 to approximately P14 and consequently change from NGF to GFL dependence (Molliver et al., 1997; Luo et al., 2007). I will refer to the neurons maintaining TrkA expression into adulthood as the late TrkA (lTrkA) population, and the neurons perinatally switching to Ret expression as the late Ret (lRet) population. Although TrkA is not expressed in the lRet population in adulthood, TrkA signaling in these cells during the prenatal period is required for initiation of Ret expression (Patel et al., 2000; Luo et al., 2007). The downregulation of TrkA as well as the upregulation of Ret require the action of transcription factors Runx1 and Tlx3 (Chen et al., 2006; Kramer et al., 2006; Yoshikawa et al., 2007; Lopes et al., 2012). In the absence of Runx1 or Tlx3, Ret expression is severely diminished and these neurons instead express TrkA and CGRP and acquire a peptidergic-like phenotype, while in the absence of downregulation of Runx1, lTrkA neurons fail to form (Chen et al., 2006; Kramer et al., 2006; Lopes et al., 2012). Runx1 and Tlx3, largely through their regulation of Ret, affect the expression of many functional molecules in lRet neurons (Chen et al., 2006; Luo et al., 2007; Golden et al., 2010; Lopes et al., 2012 and Paper IV). However, binding of IB4, which normally occurs in lRet neurons, is independent of Runx1 and Ret (Chen et al., 2006 and Paper IV) indicating that Runx1 is insufficient to completely change the identity of the neurons.

1.1.5.3 The late TrkA population

The neurons that downregulate Runx1 keep expression of TrkA until adult age and characteristically express neuropeptides Substance P and CGRP. These and other neuropeptides are released peripherally from neurons upon nociceptive and inflammatory stimuli and mediate the axon reflex of vasodilatation and plasma extravasation leading to neurogenic inflammation (reviewed by Yaprak, 2008; Chiu et al., 2012). Notably neither TrkA nor CGRP are specific markers for the lTrkA population, since both are expressed in subsets of myelinated NF200+ neurons (see Paper I and Ruscheweyh et al., 2007). Other genes primarily expressed in the lTrkA population include the heat and capsaicin receptor TrpV1, the µ opioid receptor, the stem cell factor receptor c-Kit and the artemin receptor GFRα3 (Orozco et al., 2001; Woodbury et al., 2004; Milenkovic et al., 2007; Scherrer et al., 2009). However, while TrpV1 expression is fairly limited to lTrkA neurons in mice, in rats TrpV1 is expressed in a majority of IB4- neurons (Woodbury et al., 2004). In terms of subpopulations expressing different combinations of markers, the lTrkA population is not as well characterized as the lRet population.

Downregulation of Runx1 is a requirement for the development of lTrkA neurons and signaling by Hepatocyte growth factor (HGF) through the Met receptor, in concert with TrkA signaling, has been shown to play a role in this process (Kramer et al., 2006; Gascon et al., 2010). Runx1 and the Met receptor form a repressive loop, where Runx1
efficiently suppresses the expression of Met and Met signaling in return suppresses Runx1 expression (Gascon et al., 2010). However, other factors seem to affect Runx1 as well, since Met knockout mice have only a small increase in Runx1 expression and TrkA expression is not affected (Gascon et al., 2010).

Considering the downregulation of TrkA in lRet neurons, besides the role of Runx1 there is limited evidence that Ret signaling might also be involved. Data from Ret knockouts show that the total number of TrkA cells is increased at P14 in the absence of Ret, but normalized in the adult indicating that the downregulation is only delayed (Luo et al., 2007; Golden et al., 2010 see also Paper IV Supplementary figure S2). However, a disturbed downregulation of TrkA has also been shown in some adult IB4+ neurons in Ret conditional knockouts, as well as in some presumptive Ret+ neurons when the Ret gene has been deleted and replaced by EGFP (Luo et al., 2007; Golden et al., 2010).

1.1.5.4 Populations of late Ret neurons

Within the lRet population separate subpopulations can be identified. Considering the prolonged period of Ret upregulation in different cells from E16.5 to P14, it is possible that different subpopulations acquire Ret expression with different timing. The most characterized subset of lRet neurons bind IB4 (Streit et al., 1986; Silverman and Kruger, 1988a; Molliver et al., 1997), however, in Paper V we demonstrated the existence of another subset that expresses Tyrosine hydroxylase (TH), as well as a large proportion of Ret+ neurons that are negative for both markers. Ret+ neurons can also be grouped according to their expression of GFRα coreceptors. At P14 the different GFRα coreceptors are minimally coexpressed (Luo et al., 2007) and can be correlated with other subgroup markers as shown in Table 2.

IB4+/Ret+ neurons can be further subdivided according to expression of G-protein coupled receptors of the Mas-related G protein-coupled receptor (Mrgpr) family (Dong et al., 2001). The largest subset of IB4+ neurons express MrgprD and continue expressing high levels of Runx1 into adulthood. These neurons function as cutaneous polymodal nociceptors that form free nerve endings in the epidermis (Zylka et al., 2003; 2005). Ablation of MrgprD+ neurons caused a striking loss of mechanical nociception, indicating that the main function of MrgprD+ neurons is related to this modality (Cavanaugh et al., 2009).

Smaller populations express genes of the MrgprA, MrgprB and MrgprC families and express Runx1 only transiently. The transient expression of Runx1 before E17.5 is, however, necessary for the specification of these neuronal populations (Chen et al., 2006; Liu et al., 2008; Samad et al., 2010), and downregulation of Runx1 after E17.5 is equally important as Runx1 exerts a repressive action on these genes (Liu et al., 2008).
The patterns of expression of MrgrpA/B/C genes differ largely between species. In the mouse, the MrgrpA and MrgrpC genes have undergone several recent duplications forming a large mixed cluster on chromosome 7, the genes of which show a large degree of coexpression (Zylka et al., 2003). MrgrpA3 is activated by itch-inducing substances chloroquine and histamine and is coexpressed with the itch-specific peptide Gastrin-releasing peptide, which is necessary for transmission of itch signals from the peripheral nervous system (PNS) to the central nervous system (CNS) (Liu et al., 2009). MrgrpB4 is expressed in a small population that innervates hair follicles and epidermis of hairy skin (Liu et al., 2007). The modality of MrgrpB4+ neurons is unknown as the only stimulus known to activate them is ATP and agonists for MrgrpB4 have not been identified (Liu et al., 2007).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Co-receptor</th>
<th>Cell size</th>
<th>Population size of all cells</th>
<th>Population size of Ret+ cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF200, MatA</td>
<td>GFRα2</td>
<td>Large</td>
<td>8 % (P0)</td>
<td>14 %</td>
<td>Bourane et al., 2009; Luo et al., 2009</td>
</tr>
<tr>
<td>IB4</td>
<td>GFRα2</td>
<td>Small-medium</td>
<td>21 %</td>
<td>37 %</td>
<td>Molliver et al., 1997; Luo et al., 2007, Paper IV</td>
</tr>
<tr>
<td>TH</td>
<td>GFRα2</td>
<td>Small</td>
<td>7 %</td>
<td>12 %</td>
<td>Li et al., 2011, Paper V</td>
</tr>
<tr>
<td>TrkA</td>
<td>GFRα3</td>
<td>Small-medium</td>
<td>21%</td>
<td>37 %</td>
<td>Orozco et al., 2001; Luo et al., 2007</td>
</tr>
<tr>
<td>unknown</td>
<td>GFRα1</td>
<td>Small-medium?</td>
<td>21%</td>
<td>37 %</td>
<td>Luo et al., 2007</td>
</tr>
</tbody>
</table>

Table 2. Subpopulations of Ret+ neurons.
Numbers for IB4+, TH+ and total Ret+ populations are data from Paper V. The proportion of NF200+/Ret+ neurons is an approximation from Luo et al. (2009). Other numbers are calculated from these. TrkA+/Ret+ neurons make up 9% of all neurons in rat DRG (Kashiba et al., 1998), but have not been quantified in the mouse. Note that GFRα3 is also expressed in a population of Ret+/TrkA+ neurons and NF200 in Ret− myelinated neurons not included here.

1.1.5.5 TrkA+/Ret− small neurons

Besides the ITrkA and lRet populations, a separate population of small cells expresses the cold and menthol receptor TrpM8 (further characterized in Paper III). The expression of TrpM8 is dependent on Runx1 and TrkA signaling during development (Chen et al., 2006; Luo et al., 2007) and 80% of TrpM8+ neurons coexpress Runx1 at P30 (Samad et al., 2010). These results indicate that the TrpM8+ population emerges from the iTrkA lineage, although postnatally TrpM8 is neither coexpressed with TrkA nor with Ret as we show in Paper III and Paper IV respectively. TrpM8, and the
population expressing it, was found through screening by calcium imaging a trigeminal ganglion transcript library for transcripts conferring cold or menthol sensitivity to HEK cells (McKemy et al., 2002). The population would have been hard to find without this clear cellular phenotype and possibly other populations of TrkA-/Ret- small neurons still await discovery.

1.2 RECEPTOR PROTEINS AND SIGNAL MODULATORS IN SENSORY NEURONS

The modalities of individual sensory neurons are determined by the functional receptor molecules expressed in the peripheral terminals of each neuron. Each sensory neuron has specialized structures and/or receptor molecules that respond to specific stimuli and convert them into electric information. Many mechanoreceptive neurons end in specialized structures in the dermis including Pacinian corpuscles, Meissner corpuscles, Ruffinian corpuscles and Merkel cell-neurite complexes (Delmas et al., 2011). These non-neuronal structures function to enhance specific types of mechanical information and transmit the information to receptor proteins on the nerve ending. Mechanoreceptors also form specialized endings surrounding hair follicles (circumferential and longitudinal lanceolate endings and touch domes), where they detect movements of the hair shaft (Delmas et al., 2011). Proprioceptive neurons, which also respond to mechanical stimuli in the form of stretching of muscles and tendons, have corresponding mechanosensory end organs known as muscle spindles and Golgi tendon organs. Nociceptors, thermoreceptors and pruriceptors (neurons mediating itch) usually end as free nerve endings in the epidermis and in internal organs of the body. These neurons express temperature-sensitive ion channels for thermosensation and various types of chemoreceptors for the detection of soluble molecules. The different functional subtypes can be related to different developmental lineages, as shown by their dependence on specific genes expressed during development (reviewed by Reed-Geaghan and Maricich, 2011). The sensitivity of each neuron is further modified by the presence of diverse modulatory ion channels and receptors. When the amount of stimuli on a nerve ending reach the threshold set by the concentration and quality of local ion channels and modulatory signals, an action potential is fired transmitting the sensory signal to the DRG and further to the spinal cord. In this chapter I will discuss some examples of sensory receptor proteins and their roles for detection of different sensory modalities.

1.2.1 Trp channels

The Transient receptor potential (Trp) ion channels are cation channels with six transmembrane domains that form homo- or heterotetrameric pores. Trp channels display a great diversity in terms of ion selectiveness and activation mechanism (Gaudet, 2007). The Trp superfamily contains seven subfamilies: TrpC (canonical or classical), TrpM (melastatin), TrpV (vanilloid), TrpML (mucolipin), TrpP (polycystin),
TrpN (no mechanoreceptor potential) and TrpA (ankyrin), however, TrpN genes have not been found in mammals (Montell, 2005). Several Trp channels from different subfamilies serve sensory functions in the visual, auditory, gustatory, olfactory or somatosensory system.

1.2.1.1 TrpM8

The TrpM "Melastatin" family was named from the founding member TrpM1, which was discovered as a tumor suppressor for melanoma (Duncan et al., 1998). The family consists of 8 members, TrpM1-8, with variable sequence similarity and function (reviewed by Fleig and Penner, 2004). In the somatosensory system, only TrpM8 has been attributed a clear role, as it responds to innocuous cold and mediates the cooling sensation of compounds like menthol and icilin (McKemy et al., 2002). As we showed in Paper III, TrpM8 is expressed in a small population of very small size DRG neurons, covering around 6% of all DRG neurons. Expression of TrpM8 in Xenopus oocytes confers them responsiveness to cooling, menthol and icilin, and TrpM8-knockout mice have severe deficits in their cellular and behavioral responses to innocuous cold and cooling compounds (McKemy et al., 2002; Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). These results demonstrate that TrpM8 is both necessary and sufficient for detection of the cooling/innocuous cold modality on a cellular level.

1.2.1.2 TrpV1

TrpV1 mediates the heat and pain sensations from capsaicin, the active component of chili peppers (Caterina et al., 1997). TrpV1 is a heat receptor that activates at noxious temperatures above 43°C in healthy animals, but is sensitized at low pH which might be relevant for heat hyperalgesia in inflammation (Tominaga et al., 1998). In the mouse, TrpV1 expression is mainly confined to ITrkA neurons and very few (5-8%) IB4+ neurons express detectable levels of TrpV1 protein, but more than one third of IB4+ neurons react to capsaicin (Woodbury et al., 2004; Hjerling-Leffler et al., 2007). In the rat TrpV1 expression is more wide-spread (Woodbury et al., 2004).

1.2.1.3 TrpA1

The TrpA1 channel has been named for its multiple N-terminal ankyrin repeats, that are believed to be essential for its covalent binding of, and activation by, many irritating or cell damaging pungent chemicals such as mustard oil, cinnamaldehyde and acrolein (tear gas) (Bandell et al., 2004; Bautista et al., 2006). Formalin also binds to and activates TrpA1, and TrpA1−/− mice show markedly decreased responses in both phases of the formalin test (McNamara et al., 2007). Similar to Aic channels (see Chapter 1.2.3 below), TrpA1 is also inhibited by amiloride (Nagata et al., 2005; Banke, 2011). There are some indications that TrpA1 also functions as a cold sensor at temperatures
below 17°C (Story et al., 2003), but TrpA1+ mice do not have alterations in cold sensitivity (Bautista et al., 2006). There also seems to be a role for TrpA1 in mechanosensation in skin as well as viscera and inner ear (Corey et al., 2004; Kwan et al., 2006; Brierley et al., 2009; Vilceanu and Stucky, 2010). In skin mechanoreception TrpA1 plays a specific role for intermediately-adapting mechanically activated currents (Brierley et al., 2011). However TrpA1 expression in heterologous cells does not confer them mechanosensitivity, indicating that other proteins are required for the mechanosensory complexes (Vilceanu and Stucky, 2010). The same might be true for other sensory modalities among the many that TrpA1 is involved in. Additional proteins are likely to be involved and the role of TrpA1 may often be merely modulatory.

The expression pattern of TrpA1 is not well understood. There is conclusive evidence that TrpA1 expression is mainly confined to small unmyelinated neurons (Kobayashi et al., 2005; Nagata et al., 2005) but the precise subpopulation(s) expressing TrpA1 is less clear. Functional experiments have shown activation via TrpA1 agonists and TrpA1-dependent mechanical sensitivity mainly in IB4+ neurons but also in a group of IB4- neurons, many of which are unresponsive to capsaicin (Hjerling-Leffler et al., 2007; Vilceanu and Stucky, 2010) indicating that they may belong to the IRet population. On the other hand, histological evidence shows that TrpA1 is largely coexpressed with CGRP, TrpV1 and GFRα3 (Story et al., 2003; Elitt et al., 2006), all markers for the ITrkA population. Furthermore, TrpA1 can be upregulated by artemin-overexpression or NGF treatment, the receptors for which are primarily expressed in the ITrkA population (Obata et al., 2005; Elitt et al., 2006). However, we (in Paper IV) and others have shown a decrease of TrpA1 upon Ret deletion (Golden et al., 2010). While this is indirect evidence and non-cell autonomous effect could regulate TrpA1 expression, on the whole it seems likely that TrpA1 is expressed in a variety of neuronal subtypes from several developmental lineages.

1.2.2 The Mas-related G protein-coupled receptor family

The Mas-related G protein-coupled receptors (Mrgrp) family is large, especially in the mouse where it has been expanded recently in evolution and now contains around 50 members (Zylka et al., 2003). Many of these have been found exclusively in the DRG, where they are expressed in specific neuronal populations and have been attributed sensory functions (Dong et al., 2001). Mrgrp gene expression in the DRG is mainly found within the IB4+ subpopulation of IRet neurons (see chapter 1.1.5.4 for a further discussion of Mrgrp expression patterns in this population), although expression of MrgrpC11 has been found to overlap considerably with the, however unspecific, ITrkA marker CGRP (Samad et al., 2010).

Ligands have been found for some Mrgrp, which give an insight into the biological functions of the receptors. MrgrpA1 and MrgrpC11 are activated by RF-amide-related neuropeptides (Han et al., 2002) while MrgrpA3 is activated by the pruritogenic
antimalarial drug chloroquine (Liu et al., 2009). MrgprD is expressed in a rather large population of cells which have been described as cutaneous polymodal nociceptors responsive to cold, heat and mechanical stimuli, with an especially central role for the mechanical pain modality in vivo (Cavanaugh et al., 2009; Rau et al., 2009). MrgprD modulates the sensitivity of neurons, presumably via binding of specific ligands. The MrgprD ligands identified today are the pruritogenic compound β-alanine, the tumor-related metabolite β-aminoisobutyric acid and the synthetic nonsteroidal estrogen analogue diethylstilbestrol (Liu et al., 2012; Uno et al., 2012).

1.2.3 Acid-sensing ion channels

Acid-sensing ion channels (Asic), or Amiloride-sensitive ion channels as the acronym has also been read, are a family of five ion channel genes, three of which have been found expressed in the DRG. Asic proteins form heteromultimeric complexes that are indeed activated by acidic pH and inhibited by the diuretic drug amiloride (Horisberger, 1998; Hesselager et al., 2004; Jones et al., 2004). However, evidence also points at a role for Asic channels for mediating mechanoreception in systems as different as muscle spindles, arteric baroreceptors and the skin (Price et al., 2001; Lu et al., 2009; Simon et al., 2010). Interestingly, deletion of all Asic genes in mouse does not decrease mechanosensitivity but instead enhances it, indicating that Asic channels are likely not direct sensors for mechanical stimuli but have a complex modulatory role (Kang et al., 2012). In line with this, deletions of single Asic genes have also been shown to cause various pain hypersensitivity phenotypes (Staniland and McMahon, 2009).

1.2.4 Piezo proteins

The sensitive detection of mechanical stimuli is a complex task, in which several proteins, including TrpA1 and members of the Asic family, have been implicated. However these proteins likely play modulatory roles in mechanosensitive neurons. The best candidates for direct mechanosensory channels known today are the mechanically gated ion channels Piezo1 and Piezo2 (encoded by the mouse genes Fam38a and Fam38b respectively) (Coste et al., 2012). Only Piezo2 is expressed in the DRG and knocking it down with small interfering RNA (siRNA) in cultured DRG neurons caused an inhibition of rapidly adapting mechanically activated currents (Coste et al., 2010). This indicates an involvement of Piezo2 in certain types of mechanoreceptors, while in others the mechanism of mechanosensation is still unknown.

1.3 SENSORY SUBPOPULATIONS IN COMPLEX SOMATOSENSATION

Besides their separate roles for detection of specific stimuli, different sensory subpopulations have also been associated with different functions in pathological conditions of the peripheral nervous system. Notions that neuropeptides were expressed
in a specific subset of sensory neurons and that these neurons also expressed the heat-sensitive channel TrpV1 lead to the association of this population with inflammatory pain. The hallmarks of inflammation, dolor (pain), calor (heat), rubor (redness) and tumor (swelling), described already in the 2nd century by Celsus, can all be related to functions of peptidergic nociceptors: heat, redness and swelling to the vasodilatation and plasma extravasation in response to neuropeptides, and pain and a sensation of heat to the thermoreceptive and nociceptive properties of these neurons. However, this doesn't mean that peptidergic neurons are the only ones transmitting those modalities.

Besides the pain caused by inflammation, a distinct type of pain is neuropathic pain induced by mechanical or metabolic insults to the sensory system, such as nerve entrapments, surgical procedures, diabetes or herpes infection. Neuropathic pain states still lack efficient treatments as non-steroidal anti-inflammatory drug (NSAID) and opioids have a limited effect. While inflammatory pain was associated with peptidergic neurons, a hypothesis was laid forward associating neuropathic pain with Ret+/IB4+ neurons (Snider and McMahon, 1998). The basis for this was a study demonstrating that phospholipase Cγ (PLCγ) expressed in the inner lamina II of the dorsal horn, an area innervated by IB4+ neurons, is central for the manifestation of neuropathic pain in mice (Malmberg et al., 1997). However, later results have shown that IB4+ neurons are also involved in inflammatory pain. For example, rats treated with IB4-saporin, a compound which binds to and selectively kills IB4+ neurons, indeed have a delayed onset of symptoms of neuropathy after nerve injury, but NGF-induced hyperalgesia (which is generally regarded as a form of inflammatory pain) is also decreased, indicating an interplay between the TrkA+ and IB4+ populations (Tarpley et al., 2004). Furthermore, conditional knockout of Runx1 at perinatal stages, when Runx1 is only expressed in a subset of IRet neurons including most of the IB4+ population, causes deficits in inflammatory but not neuropathic pain, opposite to the model described earlier (Samad et al., 2010). Thus clearly, distinct populations play distinct roles in the establishment of pain states, but explaining one pain state by the actions of one single population is an oversimplification. These complex pain states are influenced by integration of sensory signals at the spinal cord level and modulation by the autonomic nervous system and the immune system (Chiu et al., 2012; Minett et al., 2012; Stemkowski and Smith, 2012).
2 RESULTS AND DISCUSSION

2.1 PAPER I: CUX2 IN SENSORY NEURON DEVELOPMENT AND FUNCTION

Cux2 is a transcription factor that functions mainly as a repressor and has four DNA-binding domains; three CUT domains and one homeodomain (Gingras et al., 2005). Cux2 is expressed in neuronal precursors and neurons in the brain and spinal cord, where it functions in regulation of cell cycle of neural progenitors and differentiation of neurons and neurite growth (Nieto et al., 2004; Iulianella et al., 2008; Cubelos et al., 2010). As Cux2 is also expressed in developing DRG (Iulianella et al., 2008), we hypothesized that it might play a similar role in the development and diversification of sensory neurons.

In this paper we demonstrated specific expression of Cux2 in early born neurons and persistence of the expression in a subset of large myelinated neurons until adult age. While in the CNS Cux2 is expressed both in precursors and mature neurons, we found it exclusively in post-mitotic neurons in the DRG. Specifically we identified Cux2 expression in eTrkA neurons, which are early-born myelinated neurons and, based on their size and expression of NF200, belong the Aδ-fiber neuronal population. This population persisted in Ngn1+/− mice, indicating that the Cux2+/TrkA+ population found in adult mice actually corresponds to early born neurons. In the TrkB+ and TrkC+ populations Cux2 was also expressed in specific subsets covering 60 % of TrkB+ and 75 % of TrkC+ cells at E15.5. The expression of Cux2 may correspond to specific functional subsets within each population, and could also be a marker of neurons that have undergone neurogenesis early, since subsets of TrkB and TrkC populations are lost in Ngn1+/− mice and hence can be believed to be born later (Ma et al., 1999).

We studied the function of Cux2 using a gene-trap mouse line with an insertion in the Cux2 gene, leading to undetectable levels of Cux2 in the DRG. This mouse line (Cux2−−) has previously been characterized with focus on the spinal cord, reporting severe alterations of the DRG size and spinal cord morphology (Iulianella et al., 2008). However, our careful analysis of the DRG at different developmental stages could not show any alterations of specific neuronal populations nor of the size of the DRG. Also, the spinal cord did not show the marked alterations described by Iulianella et al. We do not know the reason for these discrepancies, although differences in genetic background may be a factor. The strain was maintained on a mixed 129SvEv/C57B6 background in both laboratories, and genetic drift over generations of inbreeding might have caused some differences.

While we showed that Cux2 does not specify Trk subtype identity of sensory neurons, we demonstrated that it is involved in the functional regulation of mechanosensitivity as Cux2−− mice are specifically hypersensitive to mechanical stimuli. It is noticeable
that Cux2 is expressed in several brain and spinal cord regions, which could possibly be involved in sensory-motor functions required for the paw withdrawal to von Frey filaments that we used as a testing paradigm. However, the normal responses to heat and cold in Cux2<sup>−/−</sup> mice indicate the absence of more general sensory-motor alterations. Also our analysis of the spinal cord did not indicate any changes of second-order neurons that would explain the phenotype. The mechanosensitive neurons affected by Cux2 loss could be part of either the TrkA<sup>+</sup>, TrkB<sup>+</sup> or TrkC<sup>+</sup> populations since all of these populations contain mechanosensory neurons. However, the paw-withdrawal test contains a nocifensive component speaking for involvement of C- or Aδ-fibers. Since TrkB and TrkC are not found in C- or Aδ-fiber neurons, it is plausible that the Cux2<sup>+/−</sup>/TrkA<sup>−</sup> Aδ neurons are accountable for the hypersensitivity phenotype.

### 2.2 PAPER II: REGULATION OF RUNX1 INDUCTION

The iT<sub>TrkA</sub> lineage of late-born small neurons gives rise to all nociceptors, thermoreceptors, pruritceptors and C-low threshold mechanoreceptors (C-LTMR) of the adult DRG. We used Runx1 as a specific marker for the iT<sub>TrkA</sub> lineage to study the factors inducing this lineage. While the role of Runx1 in the development and diversification of iT<sub>TrkA</sub> neurons has been well studied (Chen et al., 2006; Kramer et al., 2006; Marmigère et al., 2006; Yoshikawa et al., 2007; Kobayashi et al., 2012), the factors governing the expression of Runx1 in sensory neurons have not been previously studied.

We showed that the induction of Runx1 expression is governed by extrinsic factors from the cellular environment. We used TrkC<sup>Cre</sup>;Is1<sup>DTA</sup> mice, in which expression of the cell toxic protein Diphtheria toxin A is activated by Cre recombinase in TrkC<sup>+</sup> cells, to demonstrate that signaling from TrkC<sup>+</sup> cells is involved in the induction of Runx1. Screening of potential signaling molecules revealed that fibroblast growth factor (FGF) and insulin-like growth factor (IGF) signaling had Runx1-inducing activity in cultured DRG. The Fgf protein family in the mouse consists of 22 proteins with diverse functions in development (Eswarakumar et al., 2005). We focused our analysis on Fgf1 and Fgf2 as we found that these factors were expressed in the developing DRG at the time of Runx1 initiation, and that they were able to induce Runx1 expression in cultured DRG explants.

FGF signaling in the DRG would be expected to require the presence of at least one of the five mouse Fgf receptors. Indeed we showed that fibroblast growth factor receptor 1 (Fgfr1) was highly expressed in the DRG at E12.5. Since other receptors could not be detected, we concluded that the Runx1-inducing activity of FGFs likely would involve Fgfr1. Therefore we used conditional knockout mice lacking Fgfr1 in the DRG (Wnt<sup>Cre</sup>;Fgfr1<sup>lox/lox</sup>, for simplicity referred to as Fgfr1 conditional knockout, Fgfr1-cKO) (Danielian et al., 1998; Trokovic et al., 2003). These mice expressed decreased levels of Runx1 at E12.5, both in terms of fewer Runx1<sup>+</sup> cells and lower intensity of Runx1 staining in the remaining positive cells. We also detected a decrease of TrkA<sup>+</sup> neurons
and a corresponding decrease of total Isl1+ neurons in Fgfr1-cKO mice at this stage, indicating a failure of neurogenesis or an increased cell death, specifically affecting late-born neurons in the absence of FGF signaling. At E14.5 the total number of neurons, as well as the expression of Runx1 and TrkA, was similar in Fgfr1-cKO mice and wild-type littermates (data not shown in Paper II), indicating that other regulatory mechanisms besides FGF signaling can compensate for the early phenotype. At E14.5 peripheral innervation has also occurred normally in Fgfr1-cKO mice and peripheral instructive as well as anti-apoptotic signals could play a role for the recovery of cells.

The partial decrease of Runx1 in Fgfr1-cKO mice indicates that local FGF signaling is one of several factors governing the early development of small neurons. It is a common phenomenon in developmental biology that several cell extrinsic signals often interact to perform a function. Besides FGF signaling, IGF signaling may be one of the pathways involved. We found that IGF1 was able to induce Runx1 expression (Paper II, Figure 3) and that its receptor Igf1r was expressed in the DRG at E12.5 (data not shown). However, Igf ligands did not seem to be expressed in DRG neurons at the relevant time point. Expression of Igf1 mRNA was low or absent (data not shown) and Igf2 was only expressed in the mesenchyme surrounding the DRG. It is possible that diffusing IGF2 could be one of the factors involved in Runx1 induction, but our results from TrkCcre;Isl1DTA mice show that local factors from TrkC+ neurons are also needed.

Whether the Runx1 expression we measure represents the first activation or an upregulation of a gene that has already been turned on can be debated. However, we consider first activation of the gene expression in vivo and fueling early expression functionally equally important. Further biochemical studies will be required to elucidate the details of the intracellular pathway activated by FGF signaling in early DRG neurons and the molecular effects of this on the Runx1 gene.

2.3 PAPER III: EXPRESSION DYNAMICS OF THE TRPM FAMILY

Considering the multiple roles of Trp channels in the somatosensory system and the relatively scarce information about the Trp family in this system, we set out to map the expression of all TrpM genes at different developmental stages in mouse sensory ganglia. We detected expression of all TrpM mRNAs except TrpM1 during development. TrpM6 was detected at very low levels (<1% of adult TrpV1 levels at all stages examined) and TrpM5 expression was found in embryos but downregulated in the adult. The highest adult expression level was found for TrpM3, followed by TrpM4, TrpM2, TrpM7 and TrpM8.

Over the last few years, after the publication of our paper, many new studies have confirmed sensory roles for these channels. TrpM3 expression has been mapped to small-size DRG neurons and evidence from knockout mice showed that it functions as a chemo- and thermosensor tuned for noxious heat (Vriens et al., 2011). TrpM2 has earlier been reported to signal in response to ADP ribose and oxidative stress, and these
function has now also been confirmed in rat DRG (Naziroğlu et al., 2011). TrpM4 has been shown to activate upon heating, but its role as a potential heat sensor in DRG neurons has not yet been investigated (Guler et al., 2002; Talavera et al., 2005). In zebrafish, TrpM7 is expressed in Rohon-Beard mechanosensory neurons where it is required for transmitting mechanosensory signals to the central synapses (Low et al., 2011). The human TrpM7 channel has also been shown to activate upon membrane stretch (Numata et al., 2007), further implicating a role for this channel in mechanoreception. Expression of TrpM5 has been reported to be exclusive for taste receptor cells and play a role for the temperature-dependence of the perception of sweet taste (Pérez et al., 2002; Zhang et al., 2003; Talavera et al., 2005), but based on our results TrpM5 may additionally play a developmental role in the DRG.

TrpM8 plays a very defined role in the DRG as a receptor for innocuous cold and cooling compounds like menthol and icilin (Peier et al., 2002). However, the exact population of cells expressing TrpM8 has not been clear. While initial studies reported a low degree of overlap between TrpM8 and other markers, later ones performed on rats showed TrpM8 in much larger populations partially overlapping with TrpV1, CGRP and NF200 expression (Peier et al., 2002; Okazawa et al., 2004; Kobayashi et al., 2005). In addition, in a bacterial artificial chromosome (BAC)-based TrpM8-EGFP mouse, TrpM8 was detected in small to medium sized neurons and a considerable coexpression with CGRP and TrpV1 was found (Takashima et al., 2007). However, we detected TrpM8 only in very small neurons and did not find coexpression between TrpM8 and any of these subtype markers. It is possible that expression patterns differ between species, with rats expressing TrpM8 more widely than mice do. BAC-based expression constructs usually do recapitulate the native expression pattern better than classical transgenic techniques relying on isolated promoters, due to the large amount of regulatory sequences included in BACs, but ectopic expression in other cells can still occur. On the other hand, we cannot exclude that yet unknown TrpM8 transcript variants that do not bind our probe may be expressed in some populations of DRG neurons, nor that TrpM8 expression levels in some populations may be too low to be detected with this probe.

2.4 PAPER IV: THE ROLE OF RET IN SENSORY NEURONS

Ret expression marks both the eRet population of large rapidly adapting mechanoreceptors and the lRet population of small neurons that upregulate Ret perinatally. GDNF signaling through Ret is necessary for the survival of Ret+ neurons (Molliver et al., 1997). However, Ret mutant mice die soon after birth due to severe disturbances of kidney and enteric nervous system development (Schuchardt et al., 1994). Since Ret expression in lRet neurons is initiated only perinatally, conditional strategies have been required to study the effects of Ret signaling on this population.

The first published conditional Ret knockout in the sensory system was made using the Wnt1^{Cre} line, that induces recombination in the dorsal spinal cord and all neural crest-
derived structures including DRG and enteric nervous system (Danielian et al., 1998). Due to the lack of Ret expression in the gut, these mice expressed a Hirschsprung-like phenotype of intestinal swelling, decreased growth and lethality within the first weeks after birth (Luo et al., 2007). We applied a different strategy using a TH^{Cre} driver line, which expresses Cre from a bicistronic transcript based on insertion of an internal ribosomal entry site (IRES) followed by Cre in the 3’ end of the Th gene (Lindeberg et al., 2004). This driver has the advantage of not affecting the enteric nervous system severely, allowing us to study the mice in adulthood. Crossing this driver line to a conditional Ret allele (Baudet et al., 2008) we showed that Ret affects the expression of a large number of genes in the DRG. Several functional receptors and ion channels were downregulated in the mutant mice, indicating that Ret signaling promotes their expression. Somewhat contradictory to this result, we found a marked hypersensitivity phenotype to mechanical, cool, and to a lesser degree, heat stimuli. However, TH and Ret are coexpressed in several other locations than the DRG, including brain regions such as the nigrostriatal system, where loss of Ret expression could possibly cause indirect effects on the somatosensory system.

Interestingly, another conditional Ret knockout was published around the same time as our TH^{Cre}-based strain (Golden et al., 2010). In this study a Nav1.8^{Cre} strain was used as a driver, which causes recombination uniquely in sensory neurons starting approximately at E17 (Stirling et al., 2005; Samad et al., 2010). Nav1.8 is predominantly expressed in unmyelinated neurons, however, a recent study has shown that recombination also occurs in a 38 % of myelinated NF200+ neurons (Shields et al., 2012). Whether the eRet large neurons are targeted by this driver has not been directly addressed, but the fact that Ret mRNA expression is decreased to as little as 1 % of control in Nav1.8^{Cre} Ret conditional knockout (cKO) mice indicates that there might be an effect on large neurons. However, some Ret+ large neurons are also clearly spared in this strain. In contrast, our TH^{Cre} Ret cKO retains 22 % of wild-type Ret mRNA levels (unpublished) and the number of large Ret+ neurons is approximately halved.

The TH^{Cre} mediated recombination is not fully efficient in the DRG, which is probably due to low expression levels of Cre from the bicistronic transcript and/or the short window of TH gene activity in many sensory neurons, a shown in Paper V. To reach a biologically significant decrease of Ret expression in DRG neurons, we used heterozygous mice carrying one recombined and one floxed allele of Ret in combination with TH^{Cre}. In our strain, Ret^{+/c} heterozygotes already had a decrease of Ret levels to 35 % of control in the DRG (unpublished), and this lack of one Ret allele might affect Ret-related functions in cells not expressing TH. The same could be expected in the Nav1.8^{Cre} Ret cKO, since these mice also carry one somatically inactivated allele (Golden et al., 2010). Also notably, the floxed allele in the Nav1.8^{Cre} Ret cKO strain is a knocked-in human Ret9, i.e. no functional Ret43 or Ret51 is transcribed in any part of the body of knockout animals of this strain.

The similarities and differences between these two Ret cKO strains can provide some insights into the roles of different subpopulations targeted. Both strains showed an
increase in TrpM8 expression, indicative of derepression of TrpM8 in the absence of Ret. In Paper IV we demonstrated the presence of a small number of TrpM8+/IB4− double labeling cells in mutant animals, representing such derepression on a cellular level. Since TrpM8 is a functional cold receptor, this misexpression could lead to misinterpretation of cold signals as pain by IB4+ polymodal nociceptors and targeting of cold signals into central pain pathways, leading to the hypersensitivity to innocuous cold that was detected in both Ret cKO strains (Golden et al., 2010 and Paper IV).

The most intriguing question is why Ret cKO mice develop a hypersensitivity phenotype. Our THCre Ret cKO mice display a marked hypersensitivity to mechanical stimuli at baseline, and the hypersensitivity was aggravated after nerve injury or inflammation. While the same mechanical sensitivity was not seen in the Nav1.8Cre based line, these mice did show elevated pain responses in both phases of the formalin test (Golden et al., 2010). We also detected a heat hypersensitivity phenotype in our mice using the sensitive tail-flick test to heat, indicating that these mice are hypersensitive to all sensory modalities tested, although the mechanical phenotype was the most pronounced. A possible interpretation is therefore that the absence of Ret causes a general central hypersensitivity phenotype, perhaps in addition to a more specific action on mechanoreceptors. While supraspinal effects could affect general pain sensitivity, the tail-flick test is considered to be mediated by spinal reflexes without modulation from higher brain centers (Irwin et al., 1951). Thus, the outcome of the heat test should not be affected by any loss of Ret expression in modulatory brain regions.

Contrary to these Ret cKO strains, GFRα2 knockout mice show less pain behavior in the second phase of the formalin test, and are hypersensitive to noxious heat and noxious cold but not mechanical stimuli (Lindfors et al., 2006). GFRα2 is expressed in both the large MafA+ Ret+ neurons and most small Ret− neurons in the adult, including the IB4+ and TH+ subpopulations (Luo et al., 2007; Li et al., 2011). These phenotypical differences upon disturbing the same signaling pathway highlight the complexity of the system. Possibly different GFRα proteins could substitute for one another during development, and small differences in expression patterns of Ret and GFRα2 could explain the discrepancy between the knockouts.

We suggested the downregulation of the G protein-coupled receptor 35 (Gpr35) as an explanation for the hypersensitivity phenotype of Ret knockouts. Gpr35 is a receptor for endogenous tryptophan metabolites, and is interestingly also activated by several metabolites of acetylsalicylic acid (Aspirin) as well as by tyrosine metabolites, the production of which is dependent on TH (Deng and Fang, 2012; Deng et al., 2012). Gpr35 activation has been shown to have an anti-nociceptive effect in vivo in mice (Ohshiro et al., 2008; Zhao et al., 2010; Cosi et al., 2011) and the loss of Gpr35 expression that we detected in many DRG neurons of Ret cKO mice might therefore explain their increased sensitivity.
Based on their fiber type and innervation pattern, the neurons induced by touch, and disruption of the tonic firing caused mechanical allodynia (Komagata et al., 2011). The expression of Kcnq4 in Ret knockout mice has not been addressed and further experiments are required to elucidate a potential role for Kcnq4 and/or other K\(^+\) channels in relation to Ret signaling in the regulation of sensory neurons. There is also much unknown about the "normality signal" theory, like why local anesthetics seldom cause a hypersensitivity phenotype like the one described by Komagata et al. (2011).
2.5 PAPER V: TH-EXPRESSING SENSORY NEURONS

In Paper IV, figure 2I, we demonstrated the presence of a large population of Ret+/IB4- small neurons, that were efficiently recombined in THCre Ret cKO mice. Very little is known about this population, but based on the efficient THCre recombination we hypothesized that this would correspond to the population of TH+ cells that has been described in adult rat and mouse DRG (Price and Mudge, 1983; Brumovsky et al., 2006). Indeed we could show that TH was expressed in 19% of Ret+/IB4- neurons. It is likely that the remaining Ret+/IB4- small neurons express higher levels of TH during development compared IB4- neurons, leading to the more efficient recombination in the IB4- subset. It is remarkable that the population of Ret+/IB4- small neurons has gone largely unnoticed by many researchers, but possibly the concept that most Ret+ neurons are IB4- stems from early studies in the rat where a larger proportion (around 80%) of Ret+ neurons seem to be IB4- (Bennett et al., 1998; Kashiba et al., 2001).

In the skin, TH+ neurons have been shown to function as C-LTMR sensitive to very light touch and exclusively innervating small zigzag and auchene hair follicles, where they form longitudinal lanceolate endings (Li et al., 2011). Expression of TH has also been detected in visceral afferents innervating the colorectum and urinary bladder and often colabeling with CGRP or NF200 (Brumovsky et al., 2012). However, the visceral afferents only project from DRG of certain axial levels, which were not included in our analysis. Also the lack of colabeling (see below) indicates that the TH+ neurons we studied belong to the cutaneous subset.

In order to characterize the TH+ population, we performed stainings for TH and sensory neuronal markers in adult mice over development. We showed that TH+ neurons in adult mice lack expression of peptidergic/TrkA markers CGRP, Substance P and TrkA but do express nonpeptidergic/IRet markers Ret and Runx1. In early development TH was expressed in a large number of cells in line with the early recombination of the lacZ reporter in ThCre;Rosa26Cre-lox-stop-lox-lacZ mice (Paper IV, figure 1). We characterized the dynamics of TH expression and showed that 60% of neurons expressed TH at E13.5 and these TH+ neurons were TrkA+/Ret+. Two different populations of TrkA+ neurons have been described around this stage: TrkA+/Runx1+ neurons constitute 88% of lumbar TrkA+ neurons at E14.5 and TrkA+/Runx1− make up 12% (Chen et al., 2006). These two populations likely correspond to the TrkA+/Cux2− iTkAk and TrkA+/Cux2+ eTrkAk populations that we described in Paper I. We found that the TrkA+/TH+ population covered 21% of total Isl1+ cells at E13.5, very similar to the size of the eTrkAk population. Therefore it is likely that TH expression during early stages is confined to the iTkAk population, in line with the predominant recombination of small neurons using the THCre driver as we showed in Paper IV.

Over prenatal development we found TH uniquely in TrkA+ cells but the population size decreased with time and at P0 we detected TH in ~7% of all cells, which was the same proportion as in adult mice. Therefore we believe that a subset of the cells expressing TH at embryonic stages maintains the expression until P0 and forms the
persistent TH⁺ population, which maintains TH expression into adult age. However, we cannot exclude that the early and late waves of TH expression, occurring at embryonic and postnatal stages, might happen in two distinct populations. At P0 the TH⁺ cells are still TrkA⁺ but half of them also coexpress Ret, indicating that they are in the process of upregulating Ret as a part of the switch from TrkA to Ret expression. This transition is finished by P14 and from that stage onwards practically all TH⁺ cells are Ret⁺/TrkA⁻.

We showed a high degree of colabeling of TH with Vglut3 (gene name Slc17a8). The Vglut family are glutamate transporters that control transportation and secretion of glutamate. Glutamate is the main neurotransmitter for sensory input to the superficial dorsal horn of the spinal cord (Yoshimura and Jessell, 1990). In the brain Vglut3 is frequently expressed in gabaergic, cholinergic and serotonic neurons (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002). A recent study has shown that Vglut3 is indeed sufficient to allow glutamatergic neurotransmission in non-glutamatergic cells, causing a fast excitatory signaling component (Weston et al., 2011). Since TH is the rate-limiting enzyme for catecholamine synthesis, the TH⁺/Vglut3⁺ neurons in the DRG might also have a mixed neurotransmitter phenotype. The production of catecholamines in the DRG has not been clearly proven since other enzymes of the catecholamine synthesis pathway have not been found in the DRG (Brumovsky et al., 2006). However, some studies have detected very low levels of dopamine in rat DRG indicating that the levels of enzyme may be below detection limits (Weil-Fugazza et al., 1993; Bertrand and Weil-Fugazza, 1995).

Vglut3 expression has been detected in 10 % to 16 % of L4-L5 DRG neurons in different studies using a Vglut3-GFP mouse that allows for sensitive detection of Vglut3⁺ cells (Seal et al., 2009; Shields et al., 2012). We found expression of TH in 6.7 ± 0.7 % of L5 DRG neurons, indicating that TH⁺ neurons might make up a subset of the Vglut3⁺ population. Indeed a very recent study showed expression of TH in a subpopulation of Vglut3⁺ neurons (Lou et al., 2013). While we didn't detect Vglut3 in many TH⁻ neurons, this could be due to the sensitivity of the in situ hybridization probe we used for detecting Vglut3.

We showed that expression of TH is dependent on Runx1, since conditional Runx1 knockout mice express TH in significantly fewer DRG neurons than wild-type mice. This is consistent with results from Lou et al. (2013), which also showed a loss of TH expression upon deletion of Runx1. Since in other conditional Runx1 knockouts survival of DRG neurons is not compromised (Chen et al., 2006; Samad et al., 2010; Lou et al., 2013), it is likely that the putative TH⁺ population is maintained but does not express TH. Runx1 is required for expression of many other subtype-specific genes, most of which are regulated through Ret. For the expression of TH, however, we showed that Ret is not needed.

To further characterize the TH⁺ neurons, we used in situ hybridization with a battery of new DRG subtype markers. We found the highest degree of coexpression between TH and the genes Tafa-4, Zfp521, Rnf112 and Fxyd6. However, the expression patterns
were only partly overlapping with around 60% of each population colabeling with the other marker. This could indicate that there are different subtypes within the TH⁺ population, or that TH⁺ neurons may be part of a larger population with variable expression of a defined set of marker genes, perhaps the Vglut3⁺ population. Further experiments are required to better characterize the expression patterns of these genes.
CONCLUSIONS AND FUTURE PERSPECTIVES

The development of specific cell types is dependent on both internal and external cues. In this thesis I have characterized some of the populations of sensory neurons of the mouse DRG and the pathways that lead to their formation.

While we are gathering increasingly more information about the different neuronal populations present in the mouse DRG, we are still far from a complete understanding. Considering that the entire Ret+/IB4– population comprising about one quarter of all DRG neurons has gone unnoticed by so many researchers, it is likely that other neuronal populations lacking known markers still await discovery. The variable size of sensory neurons can also make discovery harder, for example the tiny TrpM8+ neurons can easily be disregarded as glia, or even artifacts, in the absence of a specific staining.

For a deep understanding of neuronal populations and the combinations of markers they express, our present histological techniques will not be sufficient. Fluorescent stainings can routinely be performed for up to four markers on one slide and specialized set-ups can handle up to about a dozen. However, resolving the coexpression patterns of the 1000 or so sensory neuron subtype markers known today is a daunting task, not to mention the discovery of novel markers. Also technical limitations in terms of different fixation requirements and the often poor quality of antibodies makes combinations difficult and results difficult to interpret. Novel high-throughput technologies such as single-cell transcriptome analysis and multiplex in situ hybridization will be valuable for obtaining unbiased overviews of mRNA expression, but for the detailed assessment of functional proteins present in different cell types we are today still bound to the use of antibodies. Efforts to create high-affinity protein-binding molecules without the need for immunization of animals have resulted in products such as Affibodies (Tolmachev et al., 2007) and Avimers (Silverman et al., 2005), but their development is still expensive and therefore focused on more lucrative clinical applications.

Genetic tracing methods, such as marker knock-in or Cre-mediated marker activation, have a specific value due to the higher stability and easier detection of fluorescent markers, than the functional proteins they substitute for. These detection techniques often show a higher degree of colabeling between different markers than classical histological techniques, indicating that technical limitations and natural variability in expression levels often blur the real activation pattern of the genes. However, the downside of genetic tracing methods is the requirement for specific mouse strains and the extensive breeding needed for combining several genetic marks.

While genetic studies have promoted the study of mouse sensory neuronal subtypes, understanding of the human sensory system is far behind. Studies on human DRG tissue are few, and the differences between rodent and human neuronal expression
profiles are not well studied. In humans, however, certain functional studies can be performed that cannot be done in mice, especially for non-noxious sensations that do not cause replicable responses in animals. Correlating function with histologically identified neuronal subpopulations is, however, a tedious work in animals and even more challenging in humans.

The issue of labeled lines versus pattern theory, discussed in the introduction, was never completely resolved. As often is the case with opposing scientific theories, neither one is sufficient to describe the reality and both contain pieces of truth. Today we have ample evidence that independent sensory neurons usually do act as labeled lines, but also that sensory information from different primary sensory neurons is integrated and modified on the way to the brain. Labeled lines can be said to crosstalk (Ma, 2010). Furthermore, there are indications that different kinds of information might be transmitted by different patterns of activity of the same primary sensory neuron. For example, Vglut2+ sensory neurons have been shown to transmit stimuli of both itch and pain dependent on the neurotransmitter released at central termini (Lagerström et al., 2010; Liu et al., 2010), and the Ret normality signal theory claims the existence of low-frequency impulses mediating fundamentally different information than high-frequency impulses in the same individual neurons (Komagata et al., 2011). The further we reach in the study of the somatosensory system, the more layers of complexity we discover that we still don't understand. Hopefully, this thesis has provided answers to some questions while opening others.
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Isaac Newton 1676
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