Discussion

This study investigated the receptive properties of mechanoreceptors and mechanonociceptors. Stimulus response functions and velocity response functions were recorded for distinct types of receptors. The characteristic response properties and mechanical latency was determined for each type of fiber. It was shown that low threshold mechanoreceptors primarily code the velocity of mechanical stimuli. The mechanical latency was in the range of tens of milliseconds for mechanoreceptors, while in unmyelinated mechanonociceptors mechanical latency reached values of hundreds of miliseconds. The period of 150 ms was the minimum time needed for the transduction of mechanical stimulus in C-fibers since the higher velocities of mechanical stimuli did not reduce the mechanical latency in those fibers. In contrast to the low threshold mechanoreceptors that were not sensitive to 8^{o}C change in temperature (24^{o}C vs. 32^{o}C), both the stimulus response and mechanical latency were temperature dependent in C-fiber mechanonociceptors. The independent analysis of C-fibers of different modality revealed that CMs are more sensitive to mechanical stimuli than CMH fibers. This detailed investigation of the mechanoreceptive properties of cutaneous sensory neurons presented the basis for further investigation of primary sensory neuron phenotype after an altered developmental circumstance.

NGF is known to be a potent regulator of nocicptive function. In this study NGF signalling was blocked during the early postnatal development using anti-NGF and this led to a permanent change in the physiology of nociceptors lasting into adulthood. The proportion of Aδ and C-fibers of different modalities was altered following neonatal NGF deprivation. The most dramatic change was a reduced noxious heat sensitivity of unmyelinated nociceptors as well as behavioral thermal hyposensitivity. NGF seems to regulate the postnatal development and determination of nociceptive phenotype of cutaneous neurons in mice. NGF may regulate the expression of a specific protein or group of proteins that normally confer noxious heat sensitivity on sensory neurons. Therefore, genes with an altered expression level in anti-NGF treated mice were screened for using gene expression microarrays. NGF regulated genes were submitted to further investigation to find those with a specific expression pattern in the DRG. *In situ*

hybridization revealed gene candidates that might be involved in setting the noxious heat sensitivity of nociceptors. *c-Kit* was one of genes with altered expression in anti-NGF treated mice and was studied in detail.

Results from this study show that mice carrying the *c-Kit* null mutation have a phenotype very similar to that observed in neonatally NGF-deprived mice. *c-Kit*$^{-/-}$ mice display profound noxious heat hyposensitivity, which is manifested as elevated thermal thresholds and reduced firing rates of heat sensitive C fibers. *c-Kit*$^{-/-}$ mice also display behavioral thermal hypoalgesia. In addition, a distinct subclass of mechanoreceptors in *c-Kit*$^{-/-}$ display hypersensitivity to mechanical stimuli. Furthermore, the c-Kit ligand, SCF, was found to potentiate the heat induced inward current in a subpopulation of small DRG neurons. Thus, c-Kit controls the transduction properties of distinct types of sensory neuron to thermal and mechanical stimuli. Other genes, identified in this screen as regulated by NGF during development, might also be involved in setting the receptive properties of primary sensory neurons.

Characteristic receptive properties of mechanoreceptors and nociceptors

The DRG contains a variety of somatic sensory mechanoreceptors that transduce mechanical stimuli. By virtue of their response thresholds, dynamic sensitivities, and adaptation properties, different mechanoreceptors are tuned to detect different aspects of somatic stimuli, such as stimulus velocity, innocuous static indentation, or noxious mechanical stimuli. The receptive properties of primary afferent neurons with myelinated or unmyelinated axons have been extensively studied (Cain et al., 2001; Koltzenburg et al., 1997; Reeh, 1986). Employment of a computer controlled stimulator enables one to precisely distinguish between the phasic and static phase of the stimuli, and therefore the phases of the receptor response. By controlling the velocity of applied stimuli it was possible to study the response properties of mechanoreceptive fibers in more detail than has hitherto been published for mouse sensory neurons.

Receptors with a static component in the response (SAM, AM and C-fibers) coded the displacement of applied stimuli. Unmyelinated afferents are known to display lower mechanosensitivity and function as nociceptors (Bessou and Perl, 1969). Separately analyzed subpopulations of C-fibers of different modalities revealed that polymodal CMH

are significantly less sensitive to higher intensity mechanical displacements compared to CM fibers (Figure 21). The lower mechanical sensitivity of CMH fibers was also reflected in a higher mechanical threshold (Table 2). While the maximal mean firing rate of CMs upon mechanical displacement is comparable to those of AMs, (9 and 11 spikes/second respectively), CMH do not fire more than on average 4 action potentials per second to suprathreshold stimuli (Figure 9). However, when stimulated with noxious heat, those fibers reach a mean maximal discharge rate of 8 spikes/second (Figure 23). This suggests that the low mechanosensitivity of CMH fibers is not due to less intrinsic excitability. Around 70% of the mechanosensitive C-fiber population are polymodal and therefore provide the major contribution to reduced firing frequency of unmyelinated nociceptors. This limited capacity of CMH to transduce mechanical stimuli into higher frequency trains of action potentials may be caused by different coupling between transduction complexes and voltage gated channels in these cells.

Low threshold mechanoreceptors (LTMRs) (RAM, D-hair, SAM) respond rapidly to the onset of the displacement and display a prominent response to the moving phase of the stimulus. Nevertheless, during the phasic response, low threshold mechanoreceptors discharge with a constant mean rate of around 50 spikes/second independently of the magnitude of the applied indentation (Figure 9). The constant firing rate might not appear obvious from the stimulus response function. The displacement response function of SAMs gives the impression that SAM fibers code for the increasing displacement because of the static component of the response at greater and longer lasting displacements (Figure 9). Similarly, due to the fast adaptation and the firing rate calculated relative to the duration of the stimulus, the RAM stimulus response curve displays decreasing firing rate for greater displacement with longer lasting tonic indentation (Figure 9). Low threshold mechanoreceptors did not code the amplitude of movement, but the velocity of the same stimulus was coded. The steepness of the velocity response function was considerable for all LTMRs (Figure 13). At slower stimulus velocities the static component was included in the SAM response due to the duration of the stimulus and accounts for the higher firing rate compared to RAMs and D-hairs at corresponding stimuli. The static component of the SAM response is most probably also the reason for the higher percentage of SAM fibers that fire AP upon the slowest probe applied. D-hairs were shown to be exquisitely sensitive to movement. These fibers detected the slowest movements and had higher firing rates for faster movements (Figure 13). It was recently shown that the high mechanical sensitivity of D-hairs may be regulated by T-type Ca^{2+} channels which act as an amplifier of electrical stimuli in these cells (Dubreuil et al., 2004; Shin et al., 2003).

The very small size and extreme inaccessibility of the mechanosensitive peripheral endings has precluded direct extracellular or intracellular recordings of electrical events evoked by mechanical stimulation of the ending in most of the mammalian afferents (Hu et al., 2006). By measuring the mechanical latency one can get an indirect insight into the processes that underlie the transduction of mechanical forces into electrical signals. The minimum mechanical latency was evoked at distinct displacements for different types of mechanoreceptor. These displacements and the corresponding mechanical latencies were termed 'characteristic'. The characteristic mechanical latency was shortest for rapidly adapting fibers (between 13 and 14 ms) and corresponded to the smallest displacement of 10 μm. SAMs displayed varying mechanical latencies. There may also exist two or more subpopulations of SAM receptors; Aβ slowly adapting type 1 and 2 afferents have been reported to have different response pattern upon stimulation (Campbell et al., 1979; Horch et al., 1974). Some studies have suggested the existence of Aβ nociceptors that may correspond to SAM receptors with longer mechanical latencies (Lawson, 2002). While LTMRs had characteristic displacements in the range of tens of micrometers, mechanonociceptive fibers needed displacements greater than 100 μm to achieve the minimum mechanical latency. Also, when stimulated with movement of greater velocitiy, and therefore greater force, all myelinated fibers displayed lower mechanical latencies that fitted with the expected theoretical values that assume a threshold displacement. The C-fibers characteristic mechanical latency was an order of magnitude higher compared to LTMRs, and required 400 μm displacement. In contrast to the myelinated receptors, stimulation at the characteristic displacement with a faster moving probe did not result in lower mechanical latency in C-fibers. Therefore, 150 ms was revealed as the minimum time that C-fiber mechanonociceptor need to generate the first AP. Unfortunately, the mechanical stimulator used in these experiments cannot move faster than used (2.9m/s); hence, it was not possible to determine an absolute limit of the mechanical latency for the low threshold mechanoreceptors.

Effect of temperature change

C-fiber mechanonociceptors displayed uniqueness in several aspects. Recording at lower temperatures affected solely the stimulus response function and mechanical latency of C-fibers, but not the other mechanosensitive receptors; C-fibers fired less and with longer mechanical latencies when recorded at 24^{o}C compared to 32^{o}C (Figure 9-10). Two analyzed parameters of mechanotransduction in C-fibers, stimulus response

and mechanical latency, had Q_{10} values 2.06 and 2.95, respectively. It was not possible to separate the effects of temperature on transduction itself (the generation of receptor current) from the subsequent processes of action potential encoding. The Q_{10} for receptor current in the spider mechanoreceptor (lyriform ogan) was shown to be around 3 (Hoger and French, 1999). Therefore, considering that mechanotransduction parameters in myelinated fibers were not affected by temperature change, their temperature coefficient was around 1. This finding indicates that unmyelinated mechanonociceptors have a specific transduction mechanism and electrical excitability properties. Several ion channels are almost exclusively expressed in nociceptive sensory neurons and play an important role in defining nociceptive function of primary afferents, for example NaV1.8 (Akopian et al., 1999). Low threshold mechanoreceptors characteristically rapidly transform mechanical forces into electrical signals. The transduction of mechanical stimuli probably takes place via the direct gating of specialized ion channels in the membrane of low threshold mechanoreceptors (Hu and Lewin, 2006). The transduction process in C-fibers might include activation of a second messenger signaling system. There is also the possibility that the open probability of a mechanically sensitive channel in C-fibers is highly temperature dependent, like in the case of TRP channels (Voets et al., 2004). It was recently shown that the majority C-fibers in mice loose the mechanical sensitivity when blocked by TTX at 30^{o}C, while they regain the activity and mechanosensitivity at temperatures lower than 25^{o}C (Zimmermann et al., 2007). Thus, it appears that at body temperature the electrical excitability upon mechanical stimulation in nociceptive fibers is mediated by several voltage gated sodium channels, but at low temperatures it depends purely on Nav1.8.

The detailed study of cutaneous primary sensory afferents revealed several new findings:

- Distinct receptor types have a characteristic mechanical latency
- Polymodal C-fiber receptors have lower mechanical sensitivity than CM fibers
- The minimum time needed for C-fiber mechanonociceptors to evoke an action potential upon mechanical stimulation is 150 ms
- The response to mechanical stimulation of C-fiber nociceptors is uniquely sensitive to changes in temperature

Primary sensory neuron phenotype is permanently changed after temporal NGF deprivation during postnatal development

Functional block of NGF signaling during the critical period of early postnatal development led to a permanent change in sensory neuron composition of the saphenous nerve without altering the total axon number (Figure 16 and 24). NGF is required for the development of nociceptive primary sensory neurons during the period of programmed cell death (Crowley et al., 1994). Also, NGF/TrkA signaling is required for proper innervation of the skin which takes place during embryonic development (Patel et al., 2000). Various approaches have been used in the past to study NGF function. Injections of NGF and sequestration of endogenous NGF by a blocking antibody was used to analyze NGF function in adult animals (Ritter et al., 1991; Ruberti et al., 2000). It was shown that NGF signaling during early postnatal life is critical for setting the nociceptive phenotype in the rat and that it did not lead to primary sensory neuron death (Lewin et al., 1992; Ritter et al., 1991). The critical period of anti-NGF treatment was shown to be from postnatal day 4 to postnatal day 11 in rat; the treatment that did not alter the total number of neurons, but did alter the relative proportion of Aδ fibers and elevated the mechanical threshold in AMs (Lewin et al., 1992). In this study we assumed that the period corresponding to the critical in rat would be covered by anti-NGF treatment in mice throughout the first two postnatal weeks. Indeed, the changes in Aδ fiber proportion previously seen in rat were also observed in mice and used as the criterion of the effectiveness of the anti-NGF treatment. The proportion of AMs was decreased as a consequence of anti-NGF neonatal treatment, from 67% to 37%, while D-hairs increased in relative proportion (Figure 16). This finding confirmed that anti-NGF treatment in mice had similar effects as observed in the rat. Therefore, this 'temporal phenotypic NGF knockout' could be used as a mouse model to study the effects of NGF deprivation.

Primary sensory neurons of the saphenous nerve were further investigated in detail. A statistically non-significant trend was seen in Aβ fibers; the relative proportion of SAMs tended to be increased at the expense of RAMs (Figure 16). TrkA is expressed by DRG neurons of variable sizes, but particularly by small neurons, while large cells with axons in the Aβ range predominantly express the TrkC receptor (Mu et al., 1993). The low

affinity neurotrophin receptor p75 is expressed in almost all neurons that express TrkA or TrkB, but is co-expressed in only 50% of TrkC-expressing neurons (Wright and Snider, 1995). Thus, it is possible that a small population of large cells that express the high affinity NGF receptor was affected, or that the NGF deprivation affected signaling through the p75 receptor. However, the significant changes were seen in Aδ and C-fibers. The proportion of C mechanoreceptive fibers was altered due to treatment. The number of CMs increased from 28% in control to 45%, while the percentage of CMH was decreased (Figure 16), similar to as previously recorded in rat (Lewin and Mendell, 1994). Nevertheless, induced changes were slightly different between the mouse and the rat. While being reduced in anti-NGF treated mice, CMH fibers of the rat after the same treatment were virtually extinguished (Lewin and Mendell, 1994). Also, neonatal NGF deprivation in rat led to the development of the new subpopulation of CM fibers with very low mechanical threshold (Lewin and Mendell, 1994). Although we did not distinguish among subpopulations of CM fibers, the CM fibers in anti-NGF treated mice generally displayed higher mechanical thresholds than in controls (Figure 20).

The most dramatic effect of anti-NGF treatment concerns the noxious heat sensitivity. Noxious heat hyposensitivity was manifested at the level of primary sensory neurons as a decreased abundance of CMH fibers and increased thermal threshold of remaining CMHs (Figure 16 and 23). NGF deprivation also resulted in behavioral thermal hyposensitivity. The withdrawal latency was 22% longer in treated mice compared with a control (Figure 25), similar increase in reaction time was seen in *TrpV1*$^{-/-}$ (Caterina et al., 2000). However, anti-NGF treatment did not change the expression of TRPV1 at the protein level (experimental observation, data not shown). One might speculate that increased thermal threshold of the CMHs or/and lower CMH abundance was directly responsible for behavioral thermal hyposensitivity in anti-NGF treated mice. It is also possible that anti-NGF treatment may change the number of axonal branches in the dorsal horn, which may also influence the behavioral thermal sensitivity (Hulsebosch et al., 1987).

CMH fibers of neonatally NGF deprived mice fired their first action potentials during the heat ramp at 5^{o}C higher temperature compared to control mice (Figure 23). TRPV1 is proposed to be the main heat transducer, but its expression level in DRG was not regulated by anti-NGF treatment (Figure 40). The null mutation of *TrpV1* seems to have less impact on heat sensitivity than neonatal NGF deprivation. For example, thermal threshold of the remaining heat sensitive C-fibers in *TrpV1*$^{-/-}$ mice is not different compared to *wt*, although they have reduced heat evoked discharge (Caterina et al.,

2000). Nevertheless, the contribution of TRPV1 to heat sensitivity is controversial. *Ex-vivo* recordings from backskin showed that heat responding neurons express neither TRPV1 nor TRPV2 (Woodbury et al., 2004). It has been known for some time that NGF acutely induces thermal hyperalgesia (Lewin et al., 1994). When overexpressed in the skin NGF induces heat sensitivity of virtually all C-fibers and produces a large increase in the response magnitude to heat (Davis et al., 1993; Stucky et al., 1999). Due to the ongoing activity of nociceptors in this model it was not possible to measure the influence on thermal threshold and also it was not possible to distinguish acute from a developmental NGF effect. Artemin overexpression in skin lowers the heat threshold by 4^{o}C and induces higher firing rates over a range of noxious temperatures, opposite to what was recorded in *c-Kit*$^{-/-}$ mice (Elitt et al., 2006). There are not many examples of genetically altered or antibody treated mice with increased thermal threshold. Behavioral analysis of anti-NGF transgenic mice that continue to synthesize anti-NGF throughout life showed lower sensitivity to thermal pain but heat sensitivity was not studied in detail (Ruberti et al., 2000). Mice with neutralized endogenous NGF have a reduced number of neurons responding to heat and the magnitude of the heat response is smaller (Bennett et al., 1998). Mice lacking the prokineticin receptor *PKR1* gene were also reported to have impaired behavioral responsiveness to noxious heat at temperatures lower than 50^{o}C, although the response of the primary sensory neurons was not recorded (Negri et al., 2006).

Anti-NGF treatment in this study addressed the postnatal development of thermal sensitivity. The phenotype of CMH fibers was affected by neonatal NGF deprivation in terms of the lower abundance of CMH and their altered noxious heat sensitivity. Except for small reductions in body weight, anti-NGF treated mice developed and behaved normally. It was not examined if the innervation of the skin developed properly. Functional sensitivity of primary sensory neurons is mediated by sensory endings in the skin. Therefore, inappropriate innervation could influence the transduction of sensory input. Nevertheless, CMH fibers did not display altered mechanosensitivity after anti-NGF treatment. It might be expected that impaired skin innervation causing reduced heat sensitivity of CMH, would also alter the transduction of mechanical stimuli. However, recordings of primary sensory neurons with reduced noxious thermal sensitivity indicate that the changes were induced at the level of protein expression of DRG cells. Thus, impaired NGF signaling probably altered the expression of genes that regulate noxious heat transduction in adult sensory neurons, either directly or by regulating the development of noxious heat sensitive neurons.

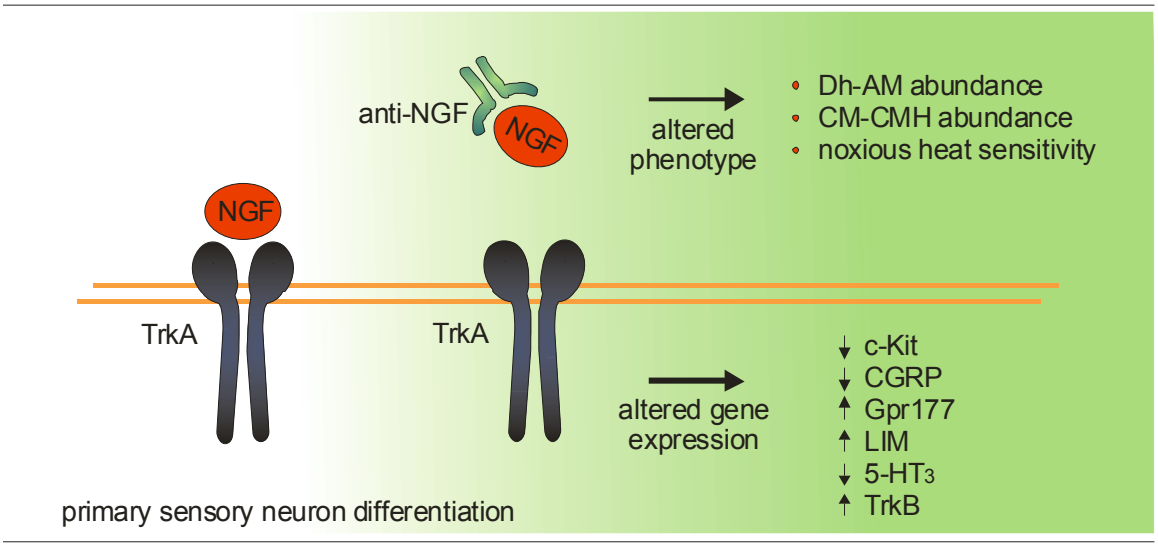

Figure 53. Schematic diagram of the changes in the phenotype of DRG primary sensory neurons and alternations in gene expression levels after temporal neonatal NGF deprivation

Anti-NGF treatment led to an increased proportion of D-hair receptors at the expense of AM fibers and to the increased proportion of CM fibers relative to the CMHs. The CMH fibers were less sensitive to the noxious heat stimulus after the neonatal NGF deprivation. Numerous genes had altered expression levels in DRG following anti-NGF treatment compared to control mice, and those changes may have caused the observed phenotypic changes.

Anti-NGF treatment regulates the postnatal development of sensory neurons influencing two aspects that determine noxious heat sensitivity. First, the number of C-fibers that are sensitive to heat was reduced (Figure 16). Higher doses of antibody might lead to even larger reduction of CMH fibers, but might also have induced sensory neuron death as have been shown previously (Ruberti et al., 2000). Second, the remaining CMH fibers are less sensitive to heat. Considering that NGF changes the c-Kit expression and that *c-Kit*$^{-/-}$ mice have a normal ratio of CM:CMH fibers, but severely impaired heat transduction, one could assume that NGF acts by two independent pathways: one that defines the CMH abundance and one that determines CMH heat sensitivity. Among C-fibers there are numerous and partially overlapping molecular markers that probably define distinct subpopulations. For example, while the majority of TrkA positive cells coexpress CGRP and substance P, cells that are GFRα3 positive also express Ret and TrkA and bind the lectin IB_4 (Orozco et al., 2001). Also, NGF acts through various signaling pathways that include ras/raf, PI-3-kinase/Akt, Ras/Map kinases, IP3/DAG, and PLCγ1 (Huang and Reichardt, 2003). NGF might be necessary for the development of some of TrkA expressing cells and influence the heat transduction of the others.

Therefore, anti-NGF treatment changed the abundance of nociceptors and their physiology and those changes may be underlined by differential gene expression (Figure 53).

Phenotypic changes after NGF deprivation may be attributable to altered gene expression

Neonatally NGF deprived mice have an altered abundance of sensory neurons of different modalities and reduced noxious heat sensitivity compared to wt mice (Figure 16 and 24). The genes that might shape the phenotype of DRG neurons in anti-NGF treated mice were expected to be differentially expressed between the anti-NGF treated group and the control group.

The phenotype that developed after neonatal NGF deprivation could be related to gene expression in several ways. NGF might be required for the development of a DRG cell of a specific modality and the abundance of each cell type in anti-NGF treated mice might be directly reflected by higher or lower expression of genes specific for such neurons. Therefore, up-regulated transcripts could correlate to D-hair mechanoreceptors or be specific to CM fibers, while down-regulated genes could be AM or CMH specific (Figure 26). Regulated genes might also be directly involved, whether higher or lower expressed, in setting noxious heat sensitivity of CMH fibers. Up-regulated genes could also be specific for GDNF receptor (Ret) expressing neurons since limited amounts of NGF might decrease the proportion of NGF dependent neurons in DRGs. Signaling molecules, whether they contribute to or inhibit physiological functions might be differently regulated.

Differentially expressed genes were identified using gene chip arrays and *in situ* hybridization. The chosen criteria to identify genes regulated in Affymetrix experiments were not very stringent for various reasons. Gene chip experiments can easily give rise to false positive and false negative results. Validation of gene chip results with in situ hybridization or qPCR experiments reported around 40 to 50% inconsistence (Kothapalli et al., 2002; Zirlinger et al., 2001). Also, individual differences between mice and the fact that the exact time of the first injection was not determined more precisely than the day (e.g. first injection could vary between P1 and P2) could have produced some variability. The phenotype after anti-NGF treatments was verified as equally effective by the AM

and D-hair receptor relative proportion, nevertheless, the effect of the treatment on C-fibers might have been variable. Finally, genes expressed in a particular type of cells specifying its physiological function could also be expressed in other types of cells which were unaffected by the treatment so that the relative change in the expression level was smaller. Gene chip experiments showed that around 250 transcripts were changed in expression in anti-NGF treated mice compared to controls. The expression in DRG was analyzed for the majority of regulated transcripts using *in situ* hybridization and level of the expression change for some of them was confirmed by real time PCR (Figure 40). Around 10% of transcripts from the gene chip experiment with an altered expression level after NGF deprivation displayed a specific expression pattern in the DRG.

Serotonin receptor

The serotonin receptor type 3, 5-HT_3, demonstrated a medium-diameter cell specific expression pattern and was down-regulated in the DRG after anti-NGF treatment (Figure 27). In the periphery, 5-HT is released in response to tissue injury from mast cells and when applied exogenously, produces hyperalgesia by a direct action on the primary afferent neuron via the 5-HT_{1A} subset of serotonin receptors (Taiwo and Levine, 1992). Nevertheless, 5-HT_3 receptors seem to play a role in the antinociceptive effect of 5-HT to mechanical acute noxious stimulus trough the modulation of spinal nociceptive transmission. Pretreatment with the 5-HT_3 receptor antagonist reverses the antinociception (paw pressure test) induced by intra thecal injection 5-HT, while intra thecal injection of the 5-HT_3 agonist induces antinociceptive effects (Bardin et al., 2000). Analysis of 5-HT_3 mutant mice showed no role for this receptor in several acute pain models but an antinociceptive role in tissue injury induced persistent pain (Zeitz et al., 2002). Here it is illustrated that expression of 5-HT_3 is regulated by the availability of NGF during postnatal development, indicating that NGF participates in the establishment of nociceptive circuits that express 5-HT_3 receptor.

Low density lipoprotein receptor family

Anti-NGF treatment regulated the level of expression of several members of the LDL receptor family. Some LDL receptors also contain a Vps10p domain, specific for family of neuronal receptors that target a variety of ligands, including neurotrophins and neuropeptides (Westergaard et al., 2004). One of them, SORL1 is expressed in medium

diameter cells in DRG and was down-regulated in the DRG after neonatal NGF deprivation (Figure 28). The genetic association of SORL1 with Alzheimer disease has recently been reported (Rogaeva et al., 2007). We have found in our group that SORL1 influences the mechanoreceptive properties of primary sensory neurons; slowly adapting fibers have increased sensitivity to mechanical stimulation in *SORL1*$^{-/-}$ mice (Hu et al., manuscript in preparation). Sortilin, another member of the LDL receptor family, acts as a co-receptor with p75 to mediate the proapoptotic signal of proNGF (Nykjaer et al., 2004). It is also expressed in the DRG and determines the receptive properties of C-fibers to both mechanical and thermal stimuli (Milenkovic et al., manuscript in preparation). The function of the NGF receptors, TrkA and p75 depends on the cellular context and members of Vps10p domain receptor family seem to play an important role in specifying signaling in different cellular contexts. In this study, the limited amount of NGF during postnatal development reduced the number of AMs in the saphenous nerve and this effect may have been mediated through SORL1 signaling. However, the exact role of SORL1 in primary sensory neurons and the interaction between NGF and SORL1 needs to be further investigated.

GPCR signaling related proteins

Several proteins involved in GPCR signaling were regulated after the anti-NGF treatment. One of those is the orphan G protein coupled receptor 177, expressed in medium size DRG cells and down-regulated in the DRG after neonatal anti-NGF treatment (Figure 33). Two proteins that mediate GPCR signaling were up-regulated and expressed in small diameter cells, Gnai2 ($G\alpha_{i2}$) and RGS4 (Figure 35). Considering the increased proportion of CM fibers in anti-NGF treated mice, both of those regulating proteins might be CM specific. $G\alpha_{i2}^{-/-}$ mice show changes in the phenotype and function of intestinal and epithelial cells and develop aberrant T-cell ontogeny (Elgbratt et al., 2007; Hornquist et al., 1997). $G\alpha_{i2}$ is implicated in the receptor dependent inhibition of adenylyl cyclases, but the influence of *Gnai2* gene deletion on the sensory system has not been studied. RGS4 is known to be expressed in neuronal tissue and has recently been proposed to be a schizophrenia susceptibility gene (So et al., 2007). RGS4 is almost entirely expressed only in the IB_4-binding GDNF responsive cells and developmental expression of RGS4 coincides with the appearance of ret expressing C-fiber neurons in the DRG (Benn et al., 2001; Costigan et al., 2003). Its expression in small DRG cells and dorsal horn suggest a possible function on central processing of painful stimuli. Recently generated *RGS4*$^{-/-}$ mice revealed subtle effect on sensorimotor

system (Grillet et al., 2005). Upregulation of RGS4 after anti-NGF treatment might be a consequence of greater abundance of c-ret expressing cells due to NGF deprivation. We performed preliminary recordings of C-fibers in *RGS4*$^{-/-}$ mouse. However, initial data are not sufficient to conclude if RGS4 deficiency changes the transduction properties of primary sensory neurons. Gpr177 is a novel gene of unknown function. The downregulation upon anti-NGF treatment and expression in medium size cells suggest its possible specificity for AM mechanonociceptors. Embryonic stem cells harboring a gene trap of this gene have been obtained and we hope to breed *Gpr177*$^{-/-}$ mice and analyze its possible function in sensory transduction or the development of specific subtypes of sensory neurons.

LIM homeobox containing protein

Testis derived transcript, *Tes*, codes a protein with a LIM-domain, characteristic of the homeobox gene mec-3 which is essential for differentiation of touch receptor neurons in *c.elegans* (Way and Chalfie, 1988). Real time PCR confirmed up-regulated expression of this gene after the anti-NGF treatment although *in situ* experiments did not show a specific expression pattern in subsets of sensory neurons (Figure 40). Other members of the LIM-homeodomain gene family (Isl-1, Rlim and Lim-3) are expressed in adult rat sensory neurons and their expression in cultured neurons can be induced by NGF (Jameson and Lillycrop, 2001). *Tes* null mutant mice were created to study its function as a tumor suppressor gene, but its role in sensory system development was not investigated (Drusco et al., 2005).

Calcitonin gene related protein

CGRP (*Calca*), coexpressed in TrkA positive neurons, is released upon primary afferent activation by noxious heat (Kessler et al., 1999). Real time PCR showed a 30% dowregulation in *Calca* after anti-NGF treatment compared to control (Figure 40). It was recently shown that differential expression of *Calca* in different mouse strains may underlie differential sensitivity to noxious heat in different mouse strains (Mogil et al., 2005). The lower expression level of *Calca* in anti-NGF treated mice could be therefore a factor that produces reduced heat sensitivity. Lower content of CGRP led to diminished discharge of CMH fibers upon heat stimulation, a trend that was also seen in anti-NGF treated mice (Mogil et al., 2005). However, considering that changes in the expression level of various genes was induced by NGF deprivation during the postnatal

development, including downregulation of *c-Kit*, down-regulated *Calca* is most probably not, or it is just partial cause of lower noxious heat sensitivity. The absence of altered thermal pain in *Calca* null mutant mice argues against CGRP function as regulator of the noxious heat sensitivity in mice (Salmon et al., 1999).

	anti-NGF	**Sorl1$^{-/-}$**	**calca$^{-/-}$**	**c-Kit$^{-/-}$**
fiber type abundance in the DRG	↓ AM, CMH ↑ D-hair, CM	no change	?	↓ RAM ↑ SAM
mechanical sensitivity	not altered	AM, SAM ↑ sensitivity	not altered	SAM ↑ sensitivity
heat sensitivity	↑ CMH heat threshold	not altered	not altered	↑ CMH heat threshold ↓ firing rate
expression pattern in the DRG		medium diameter	small diameter (TrkA expressing)	small diameter (predominately TrkA expressing), and some big

Table 6. Similarities and differences between the phenotype of the neonatally NGF deprived mice and SorL1, calca and c-Kit null mutants.
SorL1, *calca* and *c-Kit* had lower expression level after anti-NGF treatment compared to the control mice.

Anti-NGF treatment led to the changed expression of numerous genes. The exact contribution of those genes to altered phenotype in anti-NGF treated mice remains to be elucidated. The specific expression pattern of some of regulated genes indicates that they may play a functional role in certain DRG neuron subpopulations (Table 6).

c-Kit controls noxious heat sensitivity

c-Kit was picked up by the Affymetrix screen as downregulated in the DRG after neonatal anti-NGF treatment. A reduction in expression level of around 25% compared to control was confirmed by qPCR (Figure 40). In addition, *c-Kit* showed a specific expression pattern in small and in some large diameter DRG cells (Figure 37). The expression of *c-Kit* mRNA in DRG has been previously reported and c-Kit receptor expression is also observed in laminae I and II of spinal cord (Hirata et al., 1995; Orr-

Urtreger et al., 1990). The survival of cultured embryonic c-Kit positive neurons can be supported by NGF, and in its presence SCF is not necessary, neither have an additive effect upon survival or neurite outgrowth (Hirata et al., 1993). Also, in the presence of SCF, NGF is not required to support the survival and development of c-Kit positive embryonic neurons in culture, where the surviving signal is mediated by the kinase activity of c-Kit (Hirata et al., 1993). The majority of c-Kit cells in DRG coexpress the TrkA receptor and CGRP, while just around 10% are c-Ret and IB4 positive (Milenkovic et al., 2007). Therefore, numerous data indicate that NGF might influence the function of c-Kit expressing cells. During postnatal development, NGF deprivation might have reduced the number of c-Kit positive cells. Nevertheless, data from cultured DRGs suggest that SCF is sufficient to support the survival of c-Kit neurons and SCF was shown to be expressed in numerous tissues including spinal cord and brain during embryogenesis, as well as epidermis in adults (Matsui et al., 1990; Milenkovic et al., 2007). The alternative possibility is that insufficient availability of NGF during anti-NGF treatment has down-regulated the expression level of c-Kit in single cells. It is difficult to speculate through which signaling pathway downregulation might occur. The CMH fibers in anti-NGF treated mice display higher heat thresholds, while the firing rate is slightly reduced. In contrast, both the heat response and the threshold are altered in *c-Kit*$^{-/-}$ mice. The absence of just one functional *c-Kit* allele is sufficient to lead to a loss of nociceptor heat sensitivity that is almost identical to that observed in *c-Kit*$^{-/-}$ mice (Figure 49). The heat threshold is affected in *c-Kit*$^{+/-}$ CMH, as well as firing rate to the noxious heat stimulus, but to a lesser degree compared to *c-Kit* null mutant. It is therefore conceivable that the amount of c-Kit expressed in the cell might control the noxious heat sensitivity. For example, it has been shown that the content of CGRP in DRG cells might influence heat sensitivity; 80% higher expression of *calca* in C57BL/6 mouse strain induces 4 fold greater response to radiant heat and 4^{o}C lower thermal threshold compared to AKR strain (Mogil et al., 2005). Therefore, this might explain why the heat sensitivity of CMH of anti-NGF treated mice that express around 75% *c-Kit* compared to wt expression level is less impaired than in *c-Kit* heterozygote or null mutant. c-Kit seems to be a very potent regulator of noxious heat sensitivity. *c-Kit* haploinsufficiency was sufficient to increase the heat threshold by 7^{o}C, while neonatally NGF deprived mice, with 25% reduced *c-Kit* expression level in the DGR, had a 5^{o}C higher heat threshold (Figure 49 and 23). However, other molecules regulated after anti-NGF treatment might also influence heat transduction, including *calca*, whose expression was 30% lowered after anti-NGF treatment (Figure 40). Also, the incidence of CMH fibers is reduced in anti-NGF treated mice. It is likely that not just one but several molecules are

involved in regulation of heat sensitivity of CMH fibers. NGF seems to control the expression of genes during DRG development that function as crucial noxious heat sensitivity regulators in unmyelinated nociceptors.

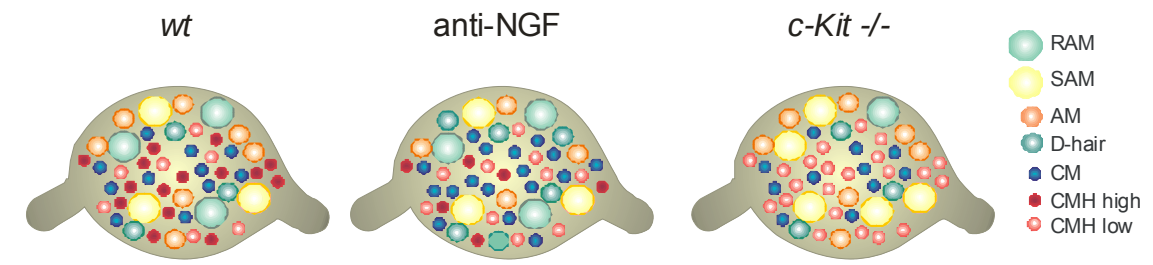

Figure 54. Schematic diagram of the abundance of distinct DRG neuron subtypes after the temporal neonatal NGF deprivation and in c-Kit null mutant.
Anti-NGF treatment led to the increased proportion of D-hair receptors (green) at the expense of AM fibers (orange) and to the increased proportion of CM fibers (blue) relative to the CMHs (high and low 'responders' are depicted in dark and pale red, respectively). In c-Kit$^{-/-}$ mice the proportion of SAMs is increased relative to RAMs, there is no change in the proportion of C-fibers, but high responding CMH fibers are missing. The partial loss of high responding CMH fibers may cause the reduced abundance of CMH fibers in neonatally deprived NGF mice.

Detailed analysis of the polymodal C-fiber phenotype in control mice and in c-Kit mutants revealed two populations of heat sensitive C-fibers - low and high heat responders (Figure 49). The incidence of heat sensitive polymodal C-fibers remains constant in *c-Kit* mutants. Following *c-Kit* deletion or *c-Kit* haploinsufficiency the high heat responders now behave like low heat responders. c-Kit might be required to functionally define a subpopulation of CMH fibers that normally fire robustly upon noxious heat stimulus (Figure 54).

A subpopulation of isolated sensory neurons in culture exhibit a specific noxious heat activated inward current, I_{heat}, which is though to be the basis of noxious heat sensitivity of polymodal nociceptors (Cesare and McNaughton, 1996). In this study I_{heat} was recorded in isolated small diameter neurons to reveal if it can be acutely modulated by SCF. Indeed, SCF was able to potentiate I_{heat} in approximately 50% primary sensory neurons (Figure 50-51). SCF sensitive neurons were observed to be those that initially displayed larger I_{heat} (Figure 51). IB_4 positive neurons have smaller noxious heat activated currents than IB_4 negative (Stucky and Lewin, 1999). This suggests that c-Kit

might be specific for subpopulation of TrkA and IB_4 negative neurons that display large I_{heat}. The SCF I_{heat} potentiation was very fast and reversible. The mechanism responsible for the potentiation remains to be discovered. It has been proposed that NGF sensitizes I_{heat} and TRPV1 by TrkA activation of PI3 kinase that leads to TRPV1 phosphorylation and increased membrane insertion of the channel (Bonnington and McNaughton, 2003; Shu and Mendell, 2001; Stein et al., 2006). Therefore it is possible that c-Kit function might be coupled with a heat sensitive ion channel, such as TRPV1. Indeed, SCF potentiates the capsaicin induced increase in Ca^{2+} intracellular concentration in around 30% of cultured DRG neurons (Milenkovic et al., 2007). Half of the c-Kit positive neurons are also TRPV1 positive, and around 30% of TRPV1 positive neurons co-express c-Kit (Milenkovic et al., 2007). The null mutation of *TrpV1* decreases the firing rate of CMH similarly to that seen in *c-Kit*$^{-/-}$ (Caterina et al., 2000). However, *TrpV1*$^{-/-}$ CMHs do not have an increased thermal threshold like in *c-Kit*$^{-/-}$. This indicates that c-Kit function may be independent of TRPV1, particularly in IB_4+ neurons where TRPV1 is not necessary for noxious heat transduction (Woodbury et al., 2004). Nevertheless, SCF/c-Kit signaling might be involved in heat hyperalgesia and this might be mediated through TRPV1 (Milenkovic et al., 2007).

In contrast to the phenotype observed after NGF deprivation where functional properties of Aβ fibers were not affected, *c-Kit* insufficiency, but not haploinsuficiency, altered the relative incidence of RAM and SAM fibers (Figure 41). This might reflect a selective cell death of the RAM population or an altered differentiation of mechanoreceptors in the absence of c-Kit signaling. There is a small (~5%), but statistically significant reduction in the number of myelinated fibers in the saphenous nerve of *c-Kit*$^{-/-}$ mutants (Milenkovic et al., in press). It is thus possible that the increased number of SAM fibers encountered was entirely caused by loss of RAM fibers. However, the physiological properties of the SAM neurons were dramatically altered in *c-Kit*$^{-/-}$ mice suggesting that the increase in SAM number could be caused by changes in mechanoreceptor properties. The numbers of spikes evoked by a series of displacement stimuli in *c-Kit*$^{-/-}$ mutants was twice that of SAM fibers recorded in control mice, and the median mechanical threshold was reduced compared to *c-Kit*$^{+/+}$ mice (Figure 43). One might hypothesize that the changes in the relative number of SAM neurons together with their hypersensitivity to mechanical stimuli might reflect a developmental role for the c-Kit receptor. For instance, c-Kit might down-regulate transduction components that set the normal mechanosensitivity of these neurons. Behavioral sensitivity to mechanical stimuli is altered in *c-Kit*$^{-/-}$ mice and this

might be ascribed to lower mechanical threshold of AM mechanonociceptors. c-Kit is known to be expressed in dorsal horn, therefore, the behavioral hypersensisitivity to mechanical stimuli could also be assigned to altered transmission of primary sensory input.

c-Kit is involved in setting the mechanical sensitivity of discrete mechanoreceptive neuronal subtypes. c-Kit signaling seems to be crucial for functional properties of heat sensitive nociceptive neurons. This study supports a model in which c-Kit activity represents the main physiological mechanism for regulation of I_{heat} and therefore regulation of noxious heat sensitivity. The signal transduction mechanisms through which activation of c-Kit regulates I_{heat} remain to be studied in detail.

Conclusions

- NGF availability influences the phenotypic development of cutaneous primary sensory neurons in mice during the first two postnatal weeks, without affecting cell survival. NGF deprivation during postnatal development permanently alters the relative incidence of nociceptive neurons of different modalities.
- NGF is crucial for the development of normal heat sensitivity. Mice deprived of NGF in early postnatal development display reduced heat sensitivity. Reduced sensitivity is underlined by a higher heat threshold of CMH fibers and their lower abundance.
- The nociceptive primary sensory neuron gene expression is partially controlled by NGF availability during postnatal development. Genes whose expression is susceptible to NGF availability are also important for setting the noxious heat sensitivity.
- SCF/c-Kit signaling has a major impact on noxious heat sensitivity. Heat sensitivity of primary sensory neurons is severely impaired in *c-Kit* null mutants. SCF can potentiate noxious heat currents.
- c-Kit is necessary to set the normal mechanical sensitivity of distinct low threshold mechanoreceptors.