NEURTURIN'S ROLE IN SENSORY NEURON PLASTICITY

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Primary sensory neurons are one of the major components of the peripheral nervous system and are required for collecting and transmitting information regarding the external and internal environment to the central nervous system (CNS). The cell bodies of these neurons are located in peripheral ganglia adjacent to the spinal cord and associated with cranial nerves V, VII, IX and X (cranial nerves I, II, VIII also carry sensory information but have specialized sensory organs that detect specific stimuli). Primary sensory neurons whose cells bodies are found in spinal ganglia (also called dorsal root ganglia (DRG)) have been the focus of intense scientific investigation because of their role in transmitting sensation including those associated with pain and as a model system for understanding mechanisms of development and plasticity. The studies presented in this dissertation focus on the interactions between primary sensory neurons and growth factors that regulate developmental events, as well as adult physiology. In particular, these studies examine sensory neurons that respond to the growth factor neurturin (NRTN), the majority of which innervate the epidermis.

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ABBREVIATIONS

AM	A-mechanical
AMH	A-mechanoheat
ARTN	artemin
ASIC	acid-sensing ion channel
ATF-3	activating transcription factor-3
BDNF	brain-derived neurotrophic factor
CC	C-cold
CCI	chronic construction injury
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CH	C-heat
CHO	Chinese hamster ovary
CM	C-mechanical
CMC	C-mechanocold
CMCH	C-mechanocold and heat
CMH	C-mechanoheat
CNS	central nervous system
CPM	polymodal C-fibers
CV	conduction velocity
DRG	dorsal root ganglia
FCS	fetal calf serum

GAP-43	growth-associated protein-43
GDNF	glial cell line-derived neurotrophic factor
GFL	glial cell line-derived neurotrophic factor family ligand
GFRα	glycosylphosphatidylinositol(GPI)-linked receptor α
HBSS	Hank's balanced salt solution
IB4	isolectin B4
ISH	*in situ* hybridization
Klf7	kruppel-like zinc finger transcription factor
KO	knock out
MAPK	mitogen-activated protein (MAP) kinase
NGF	nerve growth factor
Ngn1	neurogenin1
NRTN	neurturin
NRTN-OE	neurturin-overexpressing
NT-3	neurotrophin-3
NT-4	neurotrophin-4
$P2X_3$	P2X purinoceptor 3
PB	phosphate buffer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C

PLC	phospholipase C
ERK	extracellular signal-regulated kinase
Ret	receptor tyrosine kinase for GDNF family ligands
RT-PCR	reverse transcriptase polymerase chain reaction
Runx1	runt domain transcription factor 1
SNL	spinal nerve ligation
Sox 11	Sry-box containing gene 11
SP	substance P
SPRR1A	small proline-rich repeat protein 1A
TG	trigeminal ganglia
Trk	tropomyosin-related kinase
TRPA1	transient receptor potential cation channel, subfamily A member 1
TRPM8	transient receptor potential cation channel melastatin receptor 8
TRPV1	transient receptor potential cation channel vanilloid receptor 1
WT	wild type

PREFACE

This dissertation would not have been completed without the tremendous support and guidance of my advisor, Dr. Brian Davis. Brian is a brilliant scientist and fantastic mentor. From the first day that I joined the lab, Brian did everything possible to ensure my success as a graduate student. He is a constant source of motivation, direction, and encouragement throughout my graduate training. Thank you so much for a wonderful graduate school experience.

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1.0 INTRODUCTION

1.1 GROWTH FACTORS

1.1.1 Neurotrophins

Neurotrophins regulate survival, development, function and plasticity of the vertebrate peripheral nervous system (Korsching, 1993; Lewin and Barde, 1996; McAllister et al., 1999; Sofroniew et al., 2001). There are four neurotrophins in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Levi-Montalcini and Hamburger, 1951; Cohen et al., 1954; Barde et al., 1982; Phillips et al., 1990; Ibanez et al., 1993). Neurotrophins are synthesized and secreted by the targets of sympathetic and sensory neurons (Hallbook, 1999). One class of neurotrophin receptors is comprised of three members of the tropomyosin-related kinase (Trk) receptor tyrosine kinase family: TrkA, TrkB and TrkC. NGF binds TrkA, BDNF and NT-4 bind TrkB, NT-3 activates TrkC and to a lesser extent, TrkA and TrkB (**Fig. 1**). p75 neurotrophin receptors(p75NTR) is a receptor that binds to all neurotrophins with a similar affinity (Rodriguez-Tebar et al., 1991). p75NTR enhances the response of Trk to neurotrophins as well as increases the specificity of the Trk receptors for particular ligands. Both TrkA and p75NTR receptors have nanomolar affinities for NGF and evidence from a variety of systems suggested they cooperate in tranducting NGF

signaling (Bibel et al., 1999; Huang and Reichardt, 2003). The expression pattern of these two receptors overlap extensively in sensory neurons (Karchewski et al., 1999; Rifkin et al., 2000), for example, in rat dorsal root ganglion (DRG) neurons, p75NTR is expressed in almost all neurons that express TrkA (Wright and Snider, 1995). In vitro, neurons coexpressing TrkA and p75NTR respond to lower concentrations of NGF than cells expressing TrkA alone (Barker and Shooter, 1994; Hantzopoulos et al., 1994). Coexpression of both receptors increases 25-fold of the association rate of NGF with TrkA (Mahadeo et al., 1994). p75NTR enhances Trk response to preferred ligands (e.g., NGF for TrkA, BDNF for TrkB) while attenuating responses to nonpreferred ligands (e.g., NT3 for TrkA). For example, TrkB is readily activated by BDNF, NT3 and NT4 in the absence of p75NTR. However, when p75NTR is coexpressed, only BDNF-induced phosphorylatin of TrkB remained unchanged, that induced by NT3 and NT4 was clearly reduced (Bibel et al., 1999). Other studies have shown that functiona-perturbing antibodies to p75NTR potentiated the NT-3 responses to TrkA, suggesting that p75NTR suppresses the ability of TrkA to respond to NT-3 (Clary and Reichardt, 1994).

Neutrophins have been shown to directly bind to and dimerize Trk receptors, which results in phosphorylation of tyrosine residues in the cytoplasmic domains of these receptors. Phosphorylation of these residues can activate intracellular cascades, which include Ras/extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI-3)/Akt kinase pathway and phospholipase C-1 (PLC-1) (reviewed in (Huang and Reichardt, 2001)). During development, peripheral targets produce a limited amount of neurotrophins that act as survival factors that are thought to match the number of surviving neurons (half of all sensory and sympathetic neurons die during ontogeny), as well as the size and innervation density of target territories (reviewed in (Huang and Reichardt, 2001)). In addition to their role in sensory neuron

development, neurotrophins are involved in neuronal regeneration and sensitization and these issues are discussed below in sections 1.3, 1.4 and 1.5.

1.1.2 Glial cell lined-derived neurotrophic factor (GDNF) family members

The GDNF family ligands (GFLs) consist of GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). The GFLs signal through a unique multicomponent receptor complex, consisting of a high affinity glycosylphosphatidylinositol (GPI)-anchored binding component (GFRα1 through GFRα4) and the receptor tyrosin kinase Ret. For each GFL, there is a preferred GFRα receptor, to which the GFL binds with highest affinity and most potently activates Ret. These preferred interactions are GDNF-GFRα1, NRTN-GFRα2, ARTN-GFRα3, PSPN-GFRα4 (Fig. 2) (Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Baloh et al., 1998). However, despite these preferential interactions, there is also clear cross-talk between the different ligand-receptor pairs, such as NRTN-GFRα1, GDNF-GFRα2 (Jing et al., 1997). PSPN does not promote survival of peripheral neurons (Milbrandt et al., 1998) and will not be discussed further.

Ret is a single-pass transmembrane protein which contains four cadherin-like repeats in the extracellular domain and a typical intracellular tyrosin kinase domain (Anders et al., 2001). Ret tyrosin kinase can be stimulated in two ways, one is by GFL in a complex with GFRα in rafts *in cis*; the second way is by outside rafts of a soluble GFL-GFRα complex *in trans* (Worley et al., 2000; Paratcha et al., 2001). Ret stimulation *in trans* consistent with the finding that GFRα receptors are expressed in some tissues without Ret. For example, in adult rat brain, GFRα2 is highly expressed in extensive regions of the cerebral cortex and spetum which show no Ret expression (Trupp et al., 1998; Yu et al., 1998). In the peripheral nervous system, Schwann cells

are a rich source of GDNF and GFRα1, particularly after nerve injury, while they express no detectable levels of Ret (Naveilhan et al., 1997; Trupp et al., 1997).

GFL/GFRα binding to the extracellular domain of Ret leads to phosphorylation of intracellular tyrosine residues that serve as high-affinity binding sites for various intracellular signaling proteins. The downstream signaling cascades activated by GFLs include Ras/ERK, the PI3/Akt kinase pathway, p38 mitogen-activated protein kinase (MAPK) and PLC-1 (reviewed in (Airaksinen and Saarma, 2002)).

As all GFLs signal through the same Ret receptor, it is of interest to know whether these growth factors trigger quantitatively or qualitatively different signaling pathway. In cultured sympathetic neurons isolated from rat superior cervical ganglia, GDNF, NRTN and ARTN, by their cognate co-receptors, induce a coordinated phosphorylation of the same four key Ret tyrosines with similar kinetics and are suggested to activate a similar profile of downstream signaling pathways (Coulpier et al., 2002). Although GFLs may induce the activation of similar downstream pathways through Ret, they have different biological effects and are discussed separately in sections 1.1.2.1, 1.1.2.2 and 1.1.2.3. More recent evidences also indicate that there are Ret-independent receptors such as neural cell adhesion molecule (NCAM) and integrin β1 which can form a complex with GFRα receptors. And different GFLs could active different downstream signaling pathway through Ret-independent receptors (Schmutzler et al., 2010, more details in section 1.1.2.4).

1.1.2.1 GDNF

GDNF is a distantly-related member of the transforming growth factor-beta superfamily. It was purified and cloned from a rat glial cell line and has been shown to enhance survival of embryonic midbrain dopaminergic neurons (Lin et al., 1993). *In vitro* experiments show that GDNF supports survival of somatosensory neurons after birth (Baudet et al., 2000). Mice that lack GDNF have decreased number of neurons in DRG, sympathetic ganglia and nodose ganglia and have deficits in the development of the kidney and enteric nervous system (Moore et al., 1996). Overexpression of GDNF in skin increased the number of small unmyelinated neurons that express Ret and bind the plant isolectin B4 (IB_4) (Zwick et al., 2002). In addition, enhanced expression of GDNF in skin increased mechanical sensitivity of IB_4-positive nociceptive afferents. The increase in mechanical sensitivity was attributed to significant increases in acid-sensing ion channels 2a (ASIC2a) and 2b (ASIC2b) and transient receptor potential (TRP) cation channel, subfamily A member 1 (TRPA1), which are mechanically-sensitive channels (Mullol et al., 1997; Price et al., 2000; Albers et al., 2006). GDNF also supports the survival of motor neurons. Between embryonic days (E) 10 and 12, GDNF is expressed at high levels in a restricted region in proximal limb buds and functions as a transient survival factor for motor neurons (Wright and Snider, 1996).

1.1.2.2 NRTN

NRTN was purified and cloned from Chinese hamster ovary (CHO) cells, and has been shown to promote the survival of nodose ganglia sensory neurons, superior cervical ganglion sympathetic neurons, and around 30% of DRG neurons *in vitro* (Kotzbauer et al., 1996). In the

peripheral nervous system, GFRα2-positive neurons are small in diameter and bind IB_4. These neurons are polymodal nociceptors which have unmyelinated axons that project to skin and mediate thermal and mechanical sensation (Lu et al., 2001; Lindfors et al., 2006; Jankowski et al., 2009a). A majority of GFRα2-positive neurons express the adenosine triphosphate (ATP) receptor P2X purinoceptor 3 ($P2X_3$) and peripherin, a marker of small diameter neurons that give rise to unmyelinated fibers (Orozco et al., 2001, Lindfors et al., 2006). Depending on the mouse strain, only a small percentage of NRTN-responsive neurons express calcitonin gene-related peptide (CGRP)(1.5%) or the TRP vanilloid receptor 1 (TRPV1) (0.4%-18.2%) (Zwick et al., 2002; Lindfors et al., 2006; Malin et al., 2006).

NRTN is required for development of sensory, parasympathetic and enteric neurons. In NRTN knock out (KO) mice, the number of GFRα2-expressing neurons is significantly reduced in DRG and trigeminal ganglion (TG), and the size of the remaining GFRα2-expressing neurons is significantly smaller (Heuckeroth et al., 1999). Whether the loss of GFRα2 is due to neuronal death is still unclear as it is possible that the reduction is due to loss of GFRα2 expression. GFRα2 KO mice have a normal number of sensory neuron somata in DRG and sensory axons in saphenous nerve (Stucky et al., 2002; Lindfors et al., 2006). However, the size of IB_4-binding unmyelinated sensory neuron somata and the density of CGRP-negative free nerve endings in the footpad skin are markedly reduced (Lindfors et al., 2006). The axon diameter of the saphenous nerve is also decreased in GFRα2 KO mice. Electrophysiological studies using dissociated DRG neurons from GFRα2 KO mice showed that NRTN/GFRα2 receptor signaling is required for noxious heat but not mechanical responses (Stucky et al., 2002). Behavioral studies showed that GFRα2 KO mice were hypersensitive to noxious cold in a tail immersion test. In the formalin

test, the KO mice showed a markedly attenuated persistent phase response, indicating a deficit in inflammatory pain responses (Lindfors et al., 2006).

1.1.2.3 ARTN

GFRα3, the specific GPI-linked receptor for ARTN, was cloned based on the sequence homology to the other members of GFRα receptor family, GFRα1 and α2 (Jing et al., 1997). ARTN was cloned as a binding ligand to GFRα3 based on the homology to GDNF and NRTN (Baloh et al., 1998). ARTN can support the survival of sensory and sympathetic neurons *in vitro* (Baloh et al., 1998) and controls the migration of sympathetic precursors *in vivo* (Nishino et al., 1999). In adult mice DRG, 20% of neurons express GFRα3. The majority of GFRα3-positive cells also express Ret (82%), TrkA (80%), CGRP (70%), TRPV1 (99%) and peripherin (97%), a marker of small diameter neurons that give rise to unmyelinated fibers (Orozco et al., 2001). Overexpression of ARTN in skin caused a 20.5% increase in DRG neuron number and an increase in the mRNA level of GFRα3, TrkA, TRPV1 and TRPA1. ARTN overexpression also increased the behavioral sensitivity to both heat and noxious cold (Elitt et al., 2006).

1.1.2.4 Alternative signaling receptors for GDNF family growth factors

The presence of GFRα receptors in many regions of the peripheral and central nervous systems (CNS) that do not express Ret suggested the existence of a Ret-independent signaling mechanism for GFL (Trupp et al., 1997; Trupp et al., 1999). Recently, the presence of Ret-independent GFL signaling has been found in sensory neurons. Integrins are a large family of

heterodimeric transmembrane glycoproteins consisting of α and β subunits, and integrin β1 along with NCAM have been implicated as potential co-receptors for GFLs. NCAM, unlike Ret, can interact directly with GDNF, but high-affinity GDNF binding and downstream signaling requires co-expression of GFRα1 receptors (Paratcha et al., 2003). Coimmunoprecipitation analysis in substantia nigra dopamine neurons demonstrated that integrin β1 could form a complex with GFRα1, suggesting integrin β1 as an alternative signaling receptor for GDNF (Cao et al., 2008). Recently, Schmutzler et al. (2010) demonstrated that the GFL members NRTN and ARTN can also signal through NCAM and integrin β1 in DRG neurons. They applied Ret siRNA, NCAM siRNA and integrin β1 siRNA in DRG cultures to knock down these receptors and measured the GFL-induced enhancement in the capsaicin-stimulated release of CGRP. They found that GDNF, NRTN and ARTN use different signaling pathways to sensitize sensory neurons. GDNF induces sensitization through the MEK/Erk1/2 pathway. NRTN induces sensitization in a Ret-dependent and a Ret-independent manner via the NCAM and integrin β1 manner through PI-3Kinase pathway. ARTN cause sensitization in a Ret-dependent manner and a Ret-independent manner via the NCAM receptor.

1.2 PRIMARY AFFERENT NEURONS

Sensory neurons are defined as those neurons that detect stimuli from either the external or internal environment and transmit this information to the CNS. Among sensory neurons, specialized populations that can detect noxious thermal, chemical and mechanical stimuli are called nociceptors. Nociceptors can be activated by intense stimuli that are potentially damaging

or damaging (noxious) to the tissues and are generally thought not to be activated by innocuous stimuli such as warming or touching, although wide-dynamic range nociceptors have been described in the viscera (Mayer and Gebhart, 1994; Traub et al., 1994) and may exist elsewhere. The consensus is that nociceptors are best distinguished by their function, and specifically by their relatively high threshold for activation, regardless of the specific stimuli to which they respond (e.g. mechanical or thermal).

1.2.1 Anatomy

Sensory neuron cell bodies are located in spinal ganglia at every vertebral level as well as in association with cranial nerves, V (trigeminal), VII (facial nerve), VIII (vestibulocochlear), IX (glossopharyngeal) and X (vagus). At all vertebral levels the spinal ganglia or DRG are located on the dorsal root. Sensory neurons innervate two types of targets: somatic targets (skin, skeletal muscle and bones) and visceral targets (the inner organs). The peripheral endings of somatic nociceptors are thought to be “free nerve endings”, which are unencapsulated and unspecialized nerve endings. Free nerve endings are most frequently found in the skin and they penetrate the epidermis and end in the stratum granulosum. The anatomy of peripheral endings of visceral nociceptors is not clear yet. Both somatic and visceral nociceptors have central termination in the dorsal horn of the spinal cord (Raja SN et al., text book of pain, chapter 1).

Cutaneous sensory neurons are usually divided on the basis of the conduction velocity (CV) of their axons into C-, Aδ- and Aβ-fibers (Light AR et al., 1993). C-fibers are unmyelinated and slow conducting afferents (CV<1m/s in mouse), whereas Aδ-fibers (CV<1-10 m/s mouse, <30m/s primates) are lightly myelinated and Aβ-fibers (CV>10 m/s rodents, >30m/s

primates) are heavily myelinated. Nociceptors are found in all classes of sensory neurons. The majority of nociceptors are probably C-fibers, as C-fibers are the most abundant of all sensory fibers (e.g. in the purely mouse cutaneous saphenous nerve, ca. 2500 of the 3000 fibers are C-fibers (my own data)). However, it should be noted that in some mammals over 50% of A-fibers can detect nociceptive stimuli (Djouhri and Lawson, 2004).

1.2.2 Chemistry

Nociceptors are very heterogeneous. They express a wide array of receptors, neurotransmitters and other markers. In addition to being divided into three types by their CV, nociceptors are also classified as "peptidergic" and "non-peptidergic", which is one the most commonly used classifications. This distinction applies primarily to C-fiber nociceptors, although peptides are also expressed in some A-fiber nociceptors. The peptides in peptidergic neurons are primarily substance P (SP) and CGRP. The non-peptidergic neurons are identified based on their expression of specific gangliosides that bind IB_4. This distinction is useful for characterizing nociceptors because, in general, these two classes of neurons have different central and peripheral projections, growth factor dependencies and different expression of other nociceptor-specific proteins (Snider and McMahon, 1998; Braz et al., 2005; Zylka et al., 2005; Chen et al., 2006) (Table 1). Non-peptidergic sensory neurons, labeled by IB_4-binding, represent 41% of DRG neurons in rat (Bennett et al., 1998) and 32.5% in mice (Zwick et al., 2002), whereas 40% of DRG neurons in rat are CGRP-positive peptidergic sensory neurons (Bennett et al., 1998). During perinatal and postnatal periods, the IB_4-binding non-peptidergic neurons down-regulate the TrkA receptor for NGF and begin to express Ret, the canonical kinase mediating the function of GFLs (but see 1.1.2.4) (Bennett et al., 1996; Molliver et al., 1997).

These two classes of nociceptors also express different nociceptive markers. In mice, TRPV1 is mainly expressed in IB_4-negative, peptidergic neurons (Zwick et al., 2002; Woodbury et al., 2004; Lawson et al., 2008), whereas the majority of the non-peptidergic neurons bind IB4 and express the ATP receptor $P2X_3$ (Lindfors et al., 2006). Other members of the TRP channel family also expressed in sensory neurons include TRP melastratin-8 (TRPM8) and TRPA1, both of which have been proposed to play a role in cool/cold detection. TRPM8 is activated by menthol and cool stimuli (<23-28°C), whereas TRPA1 responds to natural compounds such as mustard and cinnamon oils and responds to colder temperatures in heterologous systems (<18°C). More detail of TRP channel expression in sensory neurons will be discussed in section 1.2.4.

1.2.3 Function

Nociceptors are best identified based on their function. Most C-fiber nociceptors are polymodal nociceptors that respond to noxious mechanical, thermal and/or chemical stimuli. These C-fibers are named C-mechanoheat (CMH) or C-mechanocold (CMC), but there are also C-mechanical (CM), C-heat (CH) and C-cold (CC) nociceptors that appear to respond preferentially to one modality (but this may change following injury or inflammation (Jankowski et al., 2009a; Jankowski et al., 2010). A-fibers are named in a similar manner; e.g., A-mechanoheat (AMH) or A-mechanical (AM).

1.2.4 TRP channels in primary afferents

TRP ion channels were first identified in a *Drosophila* phototransduction mutant that exhibited a transient instead of a sustained response to bright light. The mutation that caused this phenotype was named TRP (transient receptor potential). TRPV1 was the first of six temperature-activated TRP ion channels to be cloned. There are four TRP channels that respond to heat (TRPV isoforms 1-4) and two TRP channels that respond to cold (TRPA1 and TRPM8) (reviewed in (Dhaka et al., 2006)). These thermo-responsive TRP channels can also be activated by various botanical compounds. Each thermo-responsive TRP channel has distinct temperature thresholds of activation ranging from noxious cold (≤17ºC) to noxious heat (≥52ºC). For example, TRPV1 responds to moderate heat (≥42ºC) and capsaicin (the activate ingredient in chili peppers), TRPV2 responds to noxious heat (≥52ºC), whereas TRPV3 and TRPV4 respond to warm temperatures in the range of 27 to 42ºC. TRPA1 is activated by noxious cold temperatures (≤17ºC) and mustard oil (the active ingredient in wasabe), whereas TRPM8 responds to innocuous cool temperatures (<28ºC) and menthol (the active ingredient in mint) (reviewed in (Dhaka et al., 2006)) (**Fig. 3**).

1.2.4.1 TRPV1

TRPV1, first identified by its responsiveness to capsaicin, is a nonselective cation channel that is activated by moderate heat (≥42ºC) and protons. In mouse DRG and trigeminal ganglia, TRPV1 is primarily expressed in small-to-medium diameter, IB_4-negative, peptidergic neurons (Caterina et al., 1997; Zwick et al., 2002).

Two independent groups generated TRPV1 KO mice (Caterina et al., 2000; Davis et al., 2000) and both saw an almost complete loss of electrophysiologic responses to noxious heat and capsaicin in cultured DRG neurons. In behavioral testing, responses to capsaicin were also absent in TRPV1 KO mice, although behavioral responses to noxious heat seemed to be mostly intact (Caterina et al., 2000). For example, Davis et al. (2000) could not detect any acute thermal differences, whereas Caterina et al. (2000) observed behavioral deficits only in acute thermal sensation at temperatures ≥ 50ºC. In a follow-up study using an *ex vivo* preparation where skin, DRG and spinal cord are maintained intact in artificial CSF, the response to noxious heat in TRPV1 KO mice was not different from wild type (WT) mice, at least with respect to CMH neurons (Woodbury et al., 2004). (Subsequent studies showed that the population of CH fibers that make up less than 10% of cutaneous afferents were missing in the TRPV1 KO mice (Lawson et al., 2008)). These data suggest that TRPV1 alone cannot account for all perception of noxious heat in normal animals.

However, TRPV1 is important in establishment of inflammatory pain. Injection of complete Freund's adjuvant (CFA) or carrageenan can induce inflammation and thermal/mechanical hyperalgesia in WT mice, whereas inflammation-induced heat hyperalgesia is absent in TRPV1 KO mice (Caterina et al., 2000; Davis et al., 2000). TRPV1 can be potentiated by a number of factors known to participate in inflammation, including NGF, bradykinin, ATP, lipids and prostaglandins that may act via protein kinases A (PKA) and C (PKC) (reviewed in (Tominaga and Caterina, 2004)). Taken together, these data clearly indicate that TRPV1 is part of a common signaling pathway used by different inflammatory modulators to produce hypersensitivity and perhaps as a therapeutic target for pain management.

1.2.4.2 TRPM8

TRPM8 is identified by its ability to response to menthol and cool temperatures (<28ºC) (McKemy et al., 2002; Peier et al., 2002). TRPM8 can also be activated by a number of cooling compounds, including icilin, WS3, spearmint and eucalyptol (McKemy et al., 2002; Bandell et al., 2004). Using *in situ* hybridization (ISH) analysis, Peier et al. (2002) reported that TRPM8 is expressed in 5-10% of adult mouse DRG neurons. These TRPM8-expressing neurons are small-diameter C-fibers and do not express TRPV1, CGRP, SP, nor bind IB_4 (Peier et al., 2002).

In 2007, three independent groups generated and characterized TRPM8 KO mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Overall, the findings from these three studies consistently provided behavioral evidence that TRPM8 is required for avoidance of innocuous cold and contributes to cold-evoked nocifensive responses. All three groups did two-temperature choice experiments and demonstrated major deficits in the avoidance of moderately cool temperatures in TRPM8 KO mice. Although Colburn et al. (2007) found that TRPM8 KO mice exhibited prolonged response escape latencies on a 0ºC cold plate, the other two groups performed similar experiments and found that WT and TRPM8 KO mice exhibited no differences at 0ºC or -1ºC. Dhaka et al. (2007) observed significantly reduced spontaneous paw licking and lifting in response to acetone-induced cold sensation in TRPM8 KO mice. Following chronic construction injury (CCI) or paw inflammation with CFA, Colburn et al. (2007) observed a robust sensitivity to acetone application in WT mice that was absent in TRPM8 KO mice. These data suggested that TRPM8 may contribute to cold allodynia experienced by some patients following nerve injury.

1.2.4.3 TRPA1

Using genomic based approaches, TRPA1 (previously known as ANKTM1) was cloned and characterized as a noxious cold-activated ion channel (Story et al., 2003). TRPA1 is activated at colder temperatures than TRPM8; in heterologous expression systems, TRPA1 is activated in a broader range of activation temperatures (12-27ºC) with an average activation threshold of 17ºC (Story et al., 2003). TRPA1 is expressed in non-myelinated C- or lightly-myelinated A-fibers lacking coexpression with neurofilament 150. TRPA1 is co-expressed with CGRP, SP and TRPV1. TRPA1 is not, however, expressed with TRPM8 (Story et al., 2003). In addition to noxious cold, TRPA1 can be activated by the pungent natural compounds present in cinnamon oil, wintergreen oil, clove oil, mustard oil and ginger (Bandell et al., 2004).

In 2006, two independent groups generated and characterized TRPA1 KO mice (Bautista et al., 2006; Kwan et al., 2006). Kwan et al. (2006) showed a substantially reduced number of mustard oil-responsive neurons using calcium imaging and reduced behavioral sensitivity to oral or injected mustard oil, whereas Bautista et al. (2006) showed the complete absence of mustard oil response behaviorally or in dissociated neurons. These groups also had conflicting results regarding the behavioral responses to noxious cold. Bautista et al. (2006) found no difference in paw lifting latency on a cold plate (0ºC) and no difference in licking and flinching responses to evaporative cooling by application of acetone to the hindpaw. However, Kwan et al. (2006) found decreased behavioral responses to cold plate (0ºC) and acetone in TRPA1 KO mice. For both tests, the differences between WT and KO mice were larger for females than for males. Thus, the method of measuring the behavioral responses and the gender of mice used may have contributed to the conflicting results from these two laboratories.

In addition to its function in cold nociception in sensory neurons, TRPA1 also contributes to cold hyperalgesia after inflammation and nerve injury. Following CFA injection in rats, the percent of DRG neurons expressing TRPA1 mRNA increased from 32% to 44% and 42% at 1 and 3 days post-CFA, respectively. By day 7 post-CFA these numbers returned to normal (Obata et al., 2005). This upregulation can be induced by injection of NGF but not GDNF. Similar increases in TRPA1 mRNA were also seen after L5 spinal nerve ligation (SNL) (Obata et al., 2005; Katsura et al., 2006). Injection of an anti-NGF antibody, a p38 MAPK inhibitor or TRPA1 antisense oligodeoxynucleotide decreased the induction of TRPA1 and prevented inflammation- and nerve injury-induced cold hyperalgesia. These results provide evidence that NGF-induced TRPA1 increase in sensory neurons via p38 activation is necessary for cold hyperalgesia following inflammation or injury.

Taken together, these studies suggest that TRPA1 contributes to the transduction of mechanical, cold and chemical stimuli in nociceptor sensory neurons. Moreover, since many afferents that express TRPA1 also express TRPV1 (Story et al., 2003), it is likely that interactions between these TRP channels play a central role in the development of inflammatory hyperalgesia.

1.2.5 ASIC channels in primary afferents

ASICs (acid sensing ion channel), initially called MDEG (mammalian degenerin), BNC1 (brain Na^+ channel 1), have been cloned in the late 90's and thereafter identified as proton-gated channels (Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Waldmann et al., 1997a). In rodents, at least six ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4)

encoded by four different genes (ACCN1-4) have been described (Garcia-Anoveros et al., 1997; Waldmann et al., 1997a).

ASICs are voltage-independent, proton-gated cation channels that mainly permeable to Na^+ ions (Waldmann et al., 1997a). Extracelluar acidification activates ASIC currents transiently. The activation threshold, pH sensitivity and current kinetics depend on the subunit composition of the channel. Among ASICs, ASIC1 and ASIC3 are the most sensitive to protons. They can be activated by very small acidifications and sense ΔpH as small as -0.4 and -0.2 units from the physiological pH 7.4 respectively. Both ASIC1 and ASIC3 have activation threshold around pH 6.5 (Yagi et al., 2006; Lingueglia, 2007; Deval et al., 2008). ASIC2a currents are activated by more pronounced acidification with activation thresholds close to pH 6.0 (Lingueglia et al., 1997; Baron et al., 2001; Baron et al., 2002).

In rodents, ASICs are widely present in the nervous system, central neurons express the ASIC1a and ASIC2 subunits (Price et al., 1996; Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Lingueglia, 2007). Whereas almost all ASIC isoforms are present in primary sensory neurons of the trigeminal, vagal and dorsal root ganglia. ASIC1, ASIC2, ASIC3 are significantly expressed in the small and medium-sized nociceptive sensory neurons that are able to detect noxious thermal, chemical and high-threshold mechanical stimuli. ASIC2 and ASIC3 are also expressed in large-diameter neurons that are low-threshold mechanoreceptors (Waldmann et al., 1997b; Waldmann et al., 1997a; Liu and Simon, 2000; Bassler et al., 2001; Garcia-Anoveros et al., 2001; Voilley et al., 2001; Alvarez de la Rosa et al., 2002; Page et al., 2005; Fukuda et al., 2006). ASICs are involved in cutaneous acid-induced pain elicited by moderated pH (up to pH 6.0). For example, acid-induced cutaneous pain in healthy human volunteers can be blocked by amiloride, a non-selective ASIC channel blocker (Ugawa et al., 2002; Jones et al., 2004). The

specific ASIC channel blocker A-317567 inhibits post-operative pain and inflammatory thermal hyperalgesia in rats (Dube et al., 2005). Also in rats, local peripheral application of non-selective ASIC blockers amiloride and benzamil reduces cutaneous inflammatory pain (Rocha-Gonzalez et al., 2009).

Several members of ASIC family have been proposed to participate in mechanosensation. ASIC2a and ASIC3 are expressed in mechanoreceptors, including specialized cutaneous mechanosensory structures like Merkel nerve endings, Meissner corpuscles, and palisades of lanceolate nerve endings surrounding the hair shaft (Price et al., 2000; Garcia-Anoveros et al., 2001; Price et al., 2001; Bounoutas and Chalfie, 2007). In ASIC2 KO mice the sensitivity of low-threshold rapidly adapting mechanoreceptors is markedly reduced (Price et al., 2000). ASIC3 KO mice showed decreased sensitivity to noxious pinch (Price et al., 2001).

1.3 SENSORY NEURON DEVELOPMENT

During development, the number of sensory neurons innervating target tissues depends on limited quantities of target-derived growth factors for survival. This competition leads to programmed cell death and nearly 50% of sensory neurons die via apoptosis during development (Davies, 1996). Early in embryonic development (E11-16), most sensory neurons express TrkA and are dependent on the neurotrophin NGF. This dependency is highlighted by loss of sensory neurons in mice lacking NGF or TrkA (Crowley et al., 1994; Smeyne et al., 1994; Silos-Santiago et al., 1995). However, this dependency switches late in development such that approximately 50% of sensory neurons begin to express Ret and become responsive to GFL members (Molliver

et al., 1997). The downregulation of TrkA and initiation of Ret expression is regulated by runt-related transcription factor 1 (Runx1) (Chen et al., 2006)

Runx1 is expressed exclusively in TrkA-positive neurons at early embryonic stages, whereas postnatally its expression is restricted to a subtype of neurons, arising from the TrkA-positive population. Runx1 is important for differentiation of the TrkA-positive population, contributing to the separation of putative nociceptors into peptidergic and non-peptidergic populations of C-fibers. Interestingly, Runx1 seems to regulate nociceptive lineage by switching from being a transcriptional activator at earlier stages to acting as a repressor at later embryonic stages. During early sensory neuron development, Runx1 activates TrkA expression. Inactivation of Runx1 in the mouse leads to a loss of TrkA-expressing trigeminal sensory neurons (Chen et al., 2006). During late embryonic and postnatal stages, Runx1 seems to act as a repressor. Initially all embryonic small neurons express Runx1 and TrkA. During postnatal development, the expression of Runx1 is extinguished in prospective TrkA-positive neurons that express CGRP and SP and do not bind IB_4. The neurons that maintain the expression of Runx1 switch off TrkA, begin to express Ret, and become non-peptidergic neurons most of which bind IB_4 (reviewed in (Marmigere and Ernfors, 2007; Woolf and Ma, 2007)). In Runx1 KO mice, no cells are lost but the number of Ret-expressing neurons is decreased by over 50% (Chen et al., 2006). Thus, Runx1 is required in the specification of TrkA- and Ret-positive nociceptor cell fates.

1.4 SENSORY NEURON PLASTICITY

Peripheral sensitization by injury or inflammation evokes functional plasticity of sensory neurons. A broad range of inflammatory mediators, including growth factors, ATP, chemokines,

cytokines and protons can sensitize sensory neurons. As a result of this sensitization, nociceptors exhibit lower thresholds and respond to innocuous stimuli (allodynia) and/or respond more robustly to noxious stimuli (hypersensitivity) (reviewed in (Woolf and Ma, 2007)). Sensitizing molecules bind to their receptors on the membrane and activate multiple intracellular signal transduction pathways in peripheral terminal that include PKC (Hucho et al., 2005), PKA (Varga et al., 2006), PI3K, MAP kinases, ERK, p38, adenyl cyclase and JNK (Doya et al., 2005; Jin and Gereau, 2006; Mizushima et al., 2007). Downstream of these signaling cascades are ion channels including TRP channels (Zhang et al., 2005a; Zhang et al., 2005b; Bautista et al., 2006), voltage-gated sodium channels, G protein-coupled inwardly-rectifying potassium channels and voltage-gated calcium channels (Kerr et al., 2001; Amaya et al., 2006) (**Fig. 4A**).

In addition to driving peripheral sensitization, inflammation activates retrograde signals in nociceptors that increase the transcription of neuropeptides (SP and CGRP), BDNF and sodium channels, and the translation/transcription of TRP channels (Neumann et al., 1996; Mannion et al., 1999; Ji et al., 2002). Furthermore, inflammation induced by growth factors such as NGF increases expression of μ-opioid receptors, enhancing sensitivity to opioids (Puehler et al., 2004). After peripheral nerve axotomy, the contact of the cell body with its terminal is disrupted. Loss of target-derived growth factors and retrogradely transported signal molecules drive activation of multiple signal-transduction pathways in the cell body that alter transcription of more than 1000 genes (Costigan et al., 2002; Xiao et al., 2002). The GFRα receptors for GFLs are differentially regulated within sensory neurons after sciatic nerve injury. The percentage of neurons that express GFRα2 is markedly reduced, whereas those of GFRα1 and α3 are increased (Bennett et al., 2000). The phenotypic switch of injured DRG neuron may have functional implication in generation of neuropathic pain (**Fig. 4B).**

1.5 SENSORY NEURON REGENERATION

Although the CNS usually can't regenerate, peripheral nerves regenerate spontaneously after injury because of activation of intrinsic growth capacity of peripheral neurons and a permissive environment. After peripheral nerve injury, the distal stump of the peripheral nerve undergoes Wallerian degeneraton. This active process results in fragmentation and disintegration of the axons and myelin sheath. Debris is removed by macrophages and Schwann cells. The proximal axons can then regenerate and re-innervate their peripheral targets, allowing functional recovery of sensory neurons (Snider et al., 2002; Makwana and Raivich, 2005).

Following axotomy, sensory neurons upregulate numerous regeneration-associated genes. A number of these genes have been show to be important for neurite outgrowth and regeneration, including c-Jun, activating transcription factor-3 (ATF-3) (Seijffers et al., 2006), SRY-box containing gene 11 (Sox 11) (Jankowski et al., 2009b), small proline-repeat protein 1A (SPRR1A) (Bonilla et al., 2002) and growth-associated protein-43 (GAP-43) (Bomze et al., 2001). ATF3, a member of ATF/CREB family of transcription factors, is induced in virtually all axotomized DRG neurons (Tsujino et al., 2000). ATF3 appears to contribute to the process of regeneration as overexpression of ATF3 increases the rate of regeneration. The expression of some growth-associated genes, such as SPRR1A and c-Jun, are increased in the non-injured neurons in ATF3-overexpressing mice, suggesting that these factors contribute to regeneration and are downstream of ATF3 (Seijffers et al., 2007).

Neurotrophic factors play critical roles in neuronal survival and regeneration after nerve injury. Following peripheral nerve injury, the supply of retrogradely transported neurotrophic factors is disrupted, leading to neuronal cell death and lack of regeneration. This process can be

reversed if the neurons regenerate and reach their peripheral targets, indicating dependence on target-derived neurotrophic factors. After nerve injury, Schwann cells of the distal stump upregulate neurotrophins such as NGF, GDNF, BDNF, NT-4 and the p75 receptor (Heumann et al., 1987; Raivich et al., 1991; Hammarberg et al., 1996; Hammarberg et al., 2000; Omura et al., 2005). Application of exogenous neurotrophic factors also increases peripheral nerve regeneration, providing further evidence of a role for neurotrophic factors in regeneration. Administration of NGF results in sustained axonal regeneration and decreases neuronal loss (Rich et al., 1989b; Rich et al., 1989a). Continuous intrathecal infusion of NGF delays the onset of regeneration without affecting the rate of regeneration, suggesting that the initial loss of NGF act as a signal regulating the onset of regeneration (Gold, 1997). Intrathecal administration of NT-3 contributes to the regeneration of neurons expressing its specific receptor, TrkC (Verge et al., 1996). Exogenous BDNF application produces an improvement in diameter and myelin thickness in regeneration axons and improves functional recovery after sciatic nerve transaction (Utley et al., 1996; Lewin et al., 1997). Intrathecal administration of GDNF reverses the decrease in IB_4-binding in DRG and prevents the slowing of CV after axotomy (Bennett et al., 1998). Taken together, these studies indicate that sensory neurons continue to be dependent on neurotrophic factors after birth.

1.6 GOALS OF THE DISSERTATION

1.6.1 Regulation of GFRα receptors following axotomy

Initial experiments in the first part of the dissertation were designed to determine the proportion of neurons expressing GFRα1-3 protein in situ (i.e. tissue sections of intact DRG) and in dissociated (i.e., cultured) neurons to determine the extent to which the phenotype of cultured neurons was representative of the *in vivo* condition. We found that the expression of all three receptors changed after dissociation. Expression of GFRα2 dramatically decreased to a level such that it was not detected 24 hours after dissociation in culture, and *in vitro* expression of GFRα1 and GFRα3 also decreased. To further investigate the nature of these changes, we determined whether application of growth factors in culture could reverse the changes of their specific receptors. Unlike GFRα1 or GFRα3, the loss of GFRα2 could not be rescued by its specific ligand, NRTN. This led us to examine the phenotypic switch of GFRα2-expressing neurons after nerve injury *in vivo* and potential regulators and mechanism for the phenotypic switch.

1.6.2 Regulation of sensory neuron plasticity in skin by NRTN

Previous studies in NRTN KO mice and GFRα2 KO mice indicate that NRTN is required for GFRα2 expression, and NRTN-GFRα2 signaling is required to detect noxious heat in sensory neurons. To better understand the function of NRTN-responsive neurons, we generated

transgenic mice that overexpressed NRTN in the skin. We hypothesized that overexpression of NRTN in skin would increase GFRα2 expression in sensory neurons and behavioral sensitivity to noxious heat.

During the initial anatomical characterization of NRTN-overexpressing (NRTN-OE) mice, two striking findings broadened the original research goals from simply investigating the role of NRTN in regulating heat sensitivity in sensory neurons. First, the mRNA level of ASIC2a was significantly increased in DRG and TG of NRTN-OE mice. This finding led to the hypothesis that NRTN might regulate sensitivity to mechanical stimuli. Second, NRTN increased both mRNA and protein level of TRPM8 in DRG neurons and TRPM8 is expressed in most GFRα2-positive neurons in NRTN-OE mice, which led to the hypothesis that NRTN might regulate responses to cold and pungent chemical stimuli. To test these hypotheses, the cold-plate behavioral test and menthol-water aversion test were performed.

Finally, to examine the functional responses of nociceptors to increased concentration of NRTN at the single cell level, calcium imaging was performed on dissociated DRG neurons that were labeled by injection of IB_4 conjugated to alexa fluor 488 (IB_4-488) into to medial-dorsal side of the hindpaw. The sensitivity of non-petidergic cutaneous afferents to the ligands for TRPM8 and TRPV1 are discussed in chapter 4.

	Peptidergic (CGRP/SP positive)	Non-peptidergic (IB4 positive)
% Of DRG Neurons (rat)	40%	40%
Spinal Cord Projection	Lamina I	Lamina II outer
Growth Factor Responsiveness	NGF	NGF (devel), GDNF family (adult)
Somatic afferents	Yes	Yes (70% of epidermal afferents)
Visceral afferents	Yes (>50%)	Some (<10%)
TRPV1 (rat/mouse)	>50% (rat and mouse)	25%/4-10%
P2X3 ATP receptor (rat)	30%	70%

Table 1. Peptidergic and non-peptidergic neurons have different central and peripheral projections, growth factor dependencies and expression of other nociceptors-specific proteins.

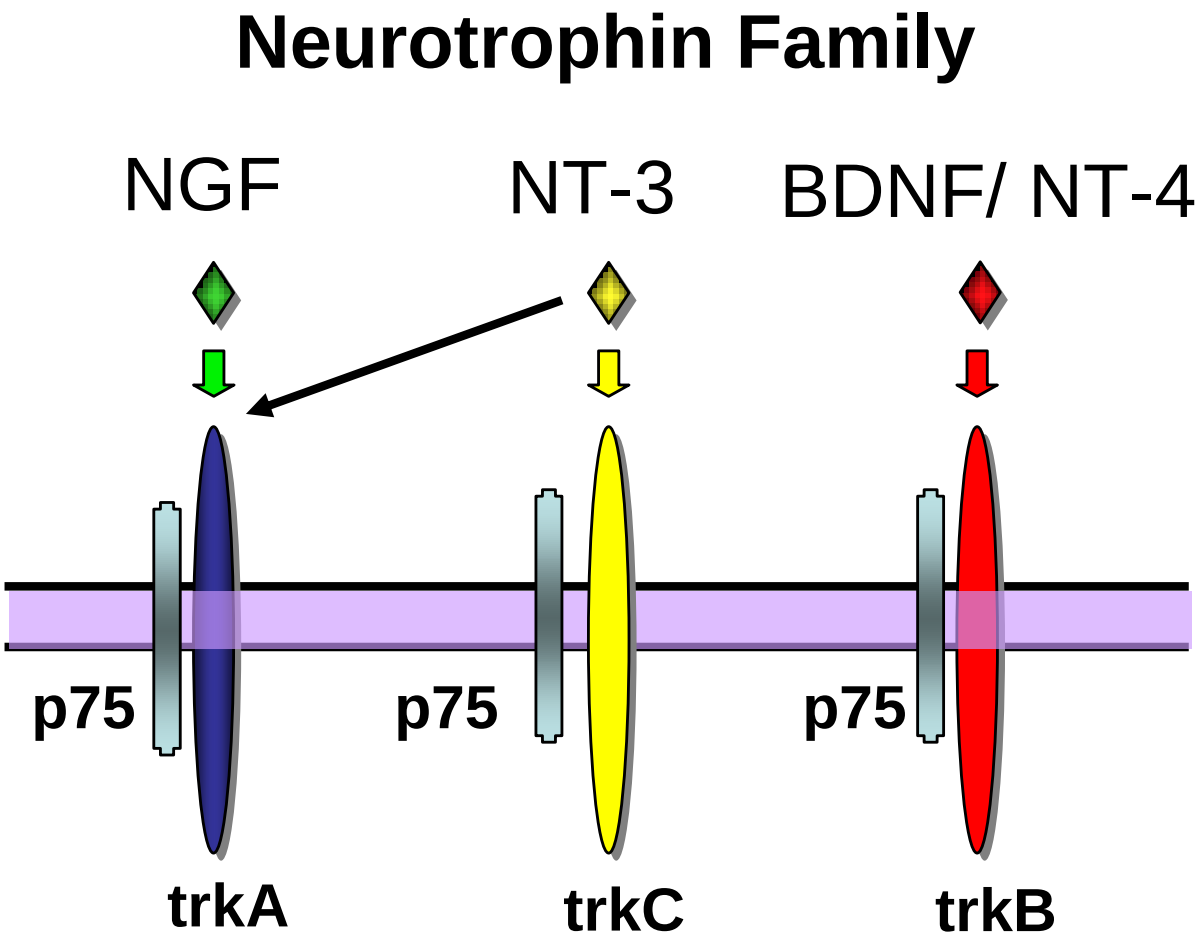


Figure 1. Neurotrophin family growth factors and their receptors.

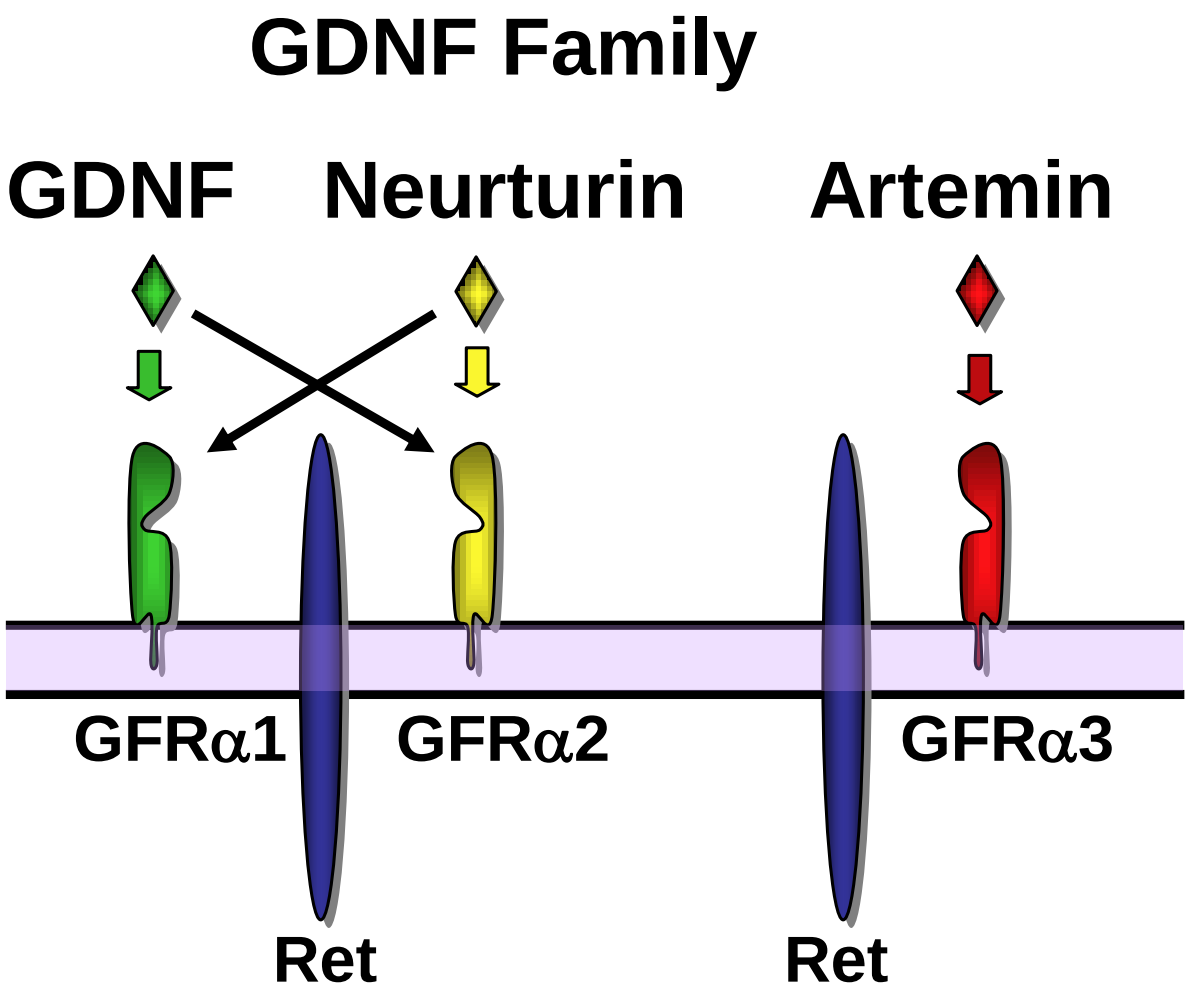


Figure 2. GDNF family growth factors and their GFRα receptors.

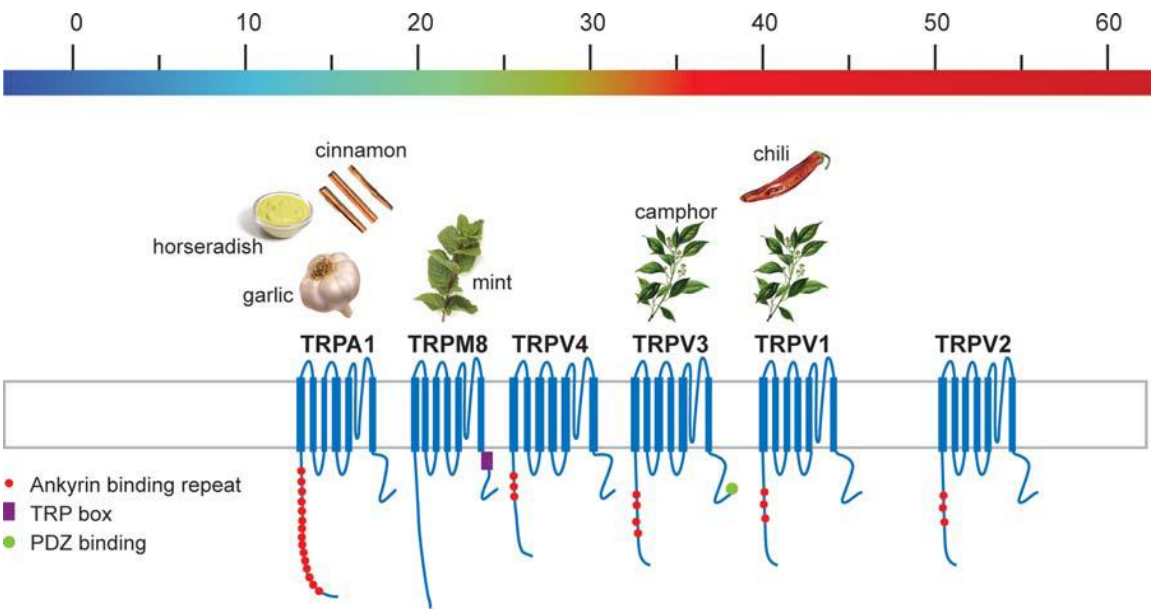


Figure 3. Thermosensitive TRP channels.

Schematic representation of the thermoTRPs that function in temperatures ranging from noxious cold to noxious heat. Proposed membrane topology and functionally important domains are represented. They include six putative transmembrane units with a proposed pore region between transmembrane domains 5 and 6. The amino and carboxy termini are cytoplasmic and contain various interaction domains like variable numbers of ankyrin repeats, TRP box or PDZ binding domains. The thermoTRPs can be activated by various botanical compounds. TRPV1 is activated by capsaicin, the pungent ingredient in chilly peppers, whereas TRPM8 is activated by menthol, the active integrate in mint. TRPA1 is activated by various pungent compounds like allicin, the active ingredient in garlic, cinnamaldehyde, the pungent component of cinnamon, and isothiocyanates, the pungent ingredients found in wasabe. Adapted from Dhaka et al., 2006.

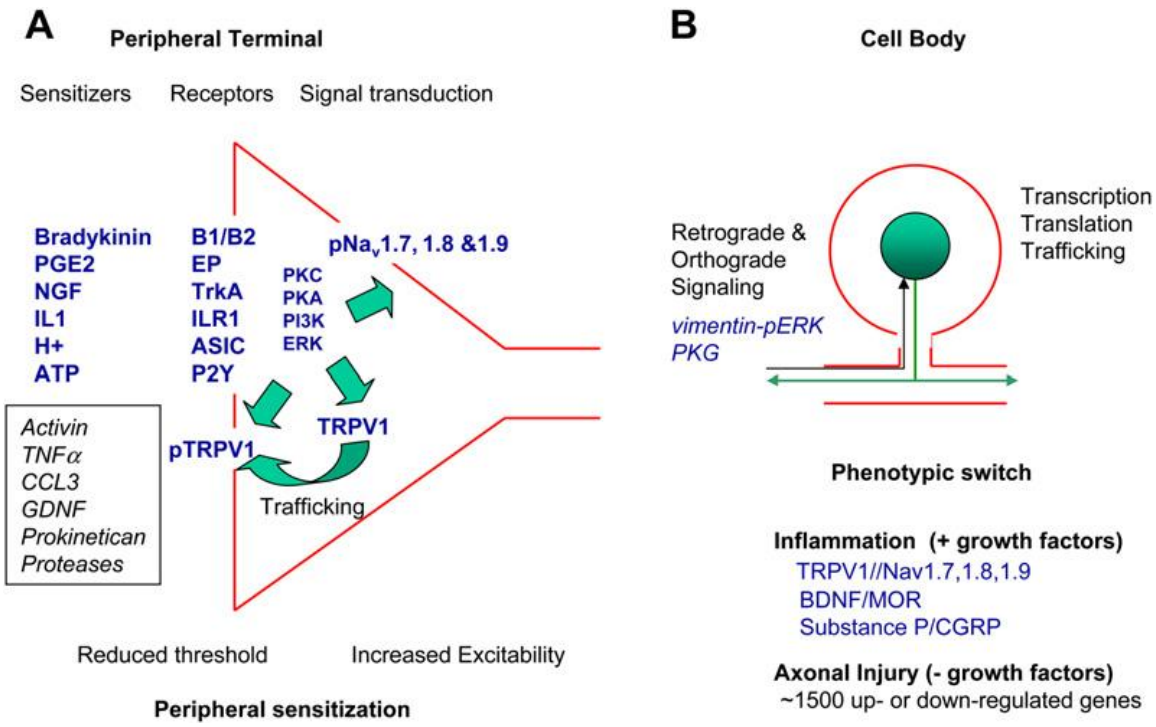


Figure 4. Nociceptor plasticity.

(A) Peripheral sensitization reduces threshold and increases excitability of nociceptors. Inflammatory sensitizers activate diverse signal transduction pathways in peripheral terminals and alter the trafficking and properties of transducer and sodium channels by phosphorylation. (B) Phenotypic switching occurs in nociceptors in response to axonal injury and inflammation due to the loss of target-derived signals or exposure to retrogradely transported signal molecules. Adapted from Woolf and Ma, 2007.

2.0 MATERIAL AND METHODS

2.1 ANIMALS

Experiments were conducted using young adult (6-8 weeks) male C57BL6/J mice (Jackson Lab) and transgenic mice that overexpress NRTN in basal keratinacytes of the skin (see below). All animals were housed in group-cages, maintained in a 12 h light/dark cycle with a temperature-controlled environment, and given food and water ad libitum. All studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

2.2 GENERATION OF TRANSGENIC MICE

Mice were generated and screened as described previously for other growth factor genes (Albers et al., 1994; Albers et al., 1996; Zwick et al., 2002; Elitt et al., 2006). A 645bp fragment that contained the coding region of NRTN was obtained by restriction digest from IMAGE Clone #5345262 (GenBank accession number BC057993) (Invitrogen), sequenced and ligated into pG4K14pro-hGH vector. A gel purified fragment containing 2.3 kb of human K14 keratin

promoter sequence, 645 bp of the mouse NRTN coding sequence, and 1.4 kb of the human growth hormone gene containing intron/exon and poly (A) signal sequences (**Fig. 16A**) was injected into C57BL7/J F1 hybrid fertilized oocytes. Founder lines were screened using slot-blot assays performed on DNA from tail skin using transgene- and NRTN-specific random primed ^{32}P-dCTP-labeled probes. Reverse transcriptase-PCR (RT-PCR) analysis of RNA from founder offspring back skin was used to assay the relative level of transgene expression. Detailed analysis was focused on the transgenic line that exhibited the highest transgene expression. Primers to detect endogenous and transgenic NRTN (5'-GGA TGT GCC AGG AGG GTC TG-3' and 5'-CAGGTCGTAGATGCGGATGG-3') as well as transgene-specific primers (5'-ACCGTGCTGTTCCGCTACTG-3' and 5'-AAGAGGGCAGCCAGTGTTTCTC-3') were used. Analyses were performed on male transgenic and wild-type (WT) mice between 4 and 8 months old.

2.3 CELL CULTURE

Primary cultures were prepared as previously described (Malin et al., 2007). Briefly, 6-8 week old male C57BL6/J mice were given an overdose of avertin anesthetic and perfused transcardially with 4°C Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS). DRG were rapidly dissected and dissociated using papain, followed by collagenase treatment. DRG were then triturated in 0.5 ml serum-containing medium, plated on laminin/poly-lysine coated dishes and fed with F12 growth media containing FCS (fetal calf serum) and antibiotics (penicillin/streptomycin, 50 U/ml).

2.4 ANIMAL SURGERY

All surgical procedures were performed under sterile conditions in a designated animal surgery area. Anesthesia was initiated by inhaled 4% isoflurane and maintained with inhaled 2% isoflurane. To assess changes in mRNA expression for GFRα1-3 and Runx1 induced by peripheral nerve axotomy, we performed sciatic and saphenous nerve axotomy. For sciatic nerve axotomy, the left hind leg was shaved, the skin was sterilized with betadine, the left sciatic nerve was exposed at the level of the head of the femur, transected and the wound was closed with wound clips. Saphenous nerve axotomy was performed on the right leg. A 5-6 mm incision was made in the skin at the mid-thigh level and the saphenous nerve was gently exposed and transected using fine scissors. Wounds were closed using 7.0 prolene sutures. At various times after surgery, mice were given an overdose of avertin anesthetic, perfused with saline (followed in some cases by 4% paraformaldehyde) and DRG L4-5 from left side (source of sciatic nerve primary afferents) or DRG L2-3 (source of saphenous nerve primary afferents) from the right side were collected.

To back-label non-peptidergic cutaneous afferents (the majority of which express GFRα2), 10µl of 2µg/µl IB_4-488 was injected subcutaneously into dorsal-medial side of both hindpaws for retrograde transport via the saphenous nerve to L2, 3 DRG. Three days later, the saphenous nerves from both sides were transected. These mice were used to identify changes of GFRα receptor expression in the IB_4-binding afferents following nerve transection. Three days after IB_4-488 injection, 1d and 6d after saphenous nerve transaction, L2, 3 DRGs from both sides were dissected for immunocytochemical staining. Three days after IB_4-488 injection, 6d after nerve injury, L2, 3 DRGs from both sides were collected and dissociated in dish to perform calcium imaging (**Fig. 5**).

2.5 IMMUNOHISTOCHEMISTRY

For *in vitro* studies, coverslips containing dissociated cells were fixed in 4% paraformaldehyde for 10 min, washed in 0.1M PBS, and then incubated in blocking solution (2% normal horse serum, 0.2% Triton X-100 in phosphate-buffered saline, pH 7.4) for 60 min. Coverslips were then incubated in primary antibodies diluted in blocking solution at 4°C overnight. Rabbit anti-TRPV1 (1:1000 Neuromics), goat anti-GFRα1 (1:500 R&D Systems), goat anti-GFRα2 (1:500 R&D Systems), goat anti-GFRα3 (1:100 R&D Systems), mouse anti-NeuN (1:100 Chemicon), rabbit anti-CGRP (1:1000 Sigma), rabbit anti-ATF3 (1:200 Santa Cruz Biotechnology), rabbit anti-PGP9.5 (1:100, Ultraclone) were used. Binding of primary antibodies was visualized with donkey anti-rabbit, donkey anti-goat or donkey anti-mouse secondary antibodies conjugated to Cy3 or Cy2 (1:1000; Jackson Immunoresearch). Coverslips were mounted in DPX on slides and photographed. Images were captured using Leica Application Suite (LAS) software and a LEICA DM 4000B microscope and using an Olympus confocal microscope.

For immunohistochemistry of IB_4-488 back-labeled DRG, mice received an overdose of avertin anesthetic followed by transcardial perfusion with 4% paraformaldehyde. DRG were collected, cryoprotected in 30% sucrose, embedded in OCT mounting medium, cut at 20µm on a cryostat and mounted on Superfrost microscope slides. Immunolabeling was performed as described above.

Both CGRP and ATF3 antibodies are generated from rabbit antiserum. To perform double-labeling with these two antibodies, we used Fab secondary antibodies as previously described (Negoescu et al., 1994). Coverslips were incubated with blocking solution at room temperature for 1 hr, incubated in CGRP antibody at 4°C overnight, washed with PBS, then incubated with goat

anti-rabbit Fab fragments (1:50; Jackson Immunoresearch) at 4°C overnight. CGRP staining was visualized using donkey anti-goat Cy3 antibody. Coverslips were then incubated with ATF3 antibody at 4°C overnight and ATF3 binding was visualized using donkey anti-rabbit Cy2 antibody.

The percentage of GFRα-positive cells (expressed as a percent of the total number of NeuN-positive cells) was calculated in L4 DRG from three mice using systematic random sampling as described previously (Malin et al., 2006). A total of 200 NeuN-positive cells per animal were assessed. Cellular profiles with a clearly defined nucleus with robust immunoreactivity (at least 5 standard deviations above background intensity) were considered positive. Images taken with one wavelength of fluorescence were scored and then overlaid with images of the second wavelength, allowing scoring of single- and double-labeled cells.

To test the specificity of TRPV1 antibody, we performed immunostaining using L4 DRG from both WT and TRPV1 KO mice (Woodbury et al., 2004). The TRPV1 staining were absent in DRG sections from TRPV1 KO mice. The specificity of TRPM8 antibody has been tested as previously described (Suzuki et al., 2007). Incubation of GST-TRPM8 antigen with TRPM8 antiserum blocked the immunostaining of TRPM8 in mouse DRG neurons. Western blots using antiserum against TRPM8 recognizes a major band at 127kDa with a lysate of DRG but not cerebral cortex which is assumed not to express TRPM8.

All GFRα antisera were purchased from R&D Systems (Minneapolis, MN) and raised in goat (GFRα1, catalog No. AF560; GFRα2, catalog No. AF429; GFRα3, catalog No. AF2645). GFRα1 antiserum was raised against recombinant rat GFRα1 extracellular domain (accession No. Q62997). The immunogen consisted of amino acid residues Asp25-Leu445 of the rat GFRα1 extracellular domain(manufacturer's technical information). This antibody is specific for GFRα1:

it blocks 50% of the binding of GDNF to immobilized GFRα1-Fc chimera in a functional enzyme-linked immunosorbent assay (ELISA); this antibody shows no cross-reactivity with Ret or GFRα3 and 10% cross-reactivity with GFRα2(manufacturer's technical information). The specificity of GFRα1 antibody staining was further confirmed by the complete absence of staining in the DRG neurons of GFRα1 KO mice (Rakowicz et al., 2002).

GFRα2 antiserum was raised against recombinant mouse GFRα2 extracellular domain (access No. NM008115). The immunogen consisted of amino acid residues Thr160-Ser441 of the GFRα2 extracellular domain (manufacturer's technical information). This antibody has been selected for its ability to neutralize receptor-ligand interation. It shows less than 5% cross-reactivity with GFRα3 in direct ELISA and western blot assays. This antibody has been previously shown to produce labeling in pelvic ganglia, DRG and brain of WT but not GFRα2 KO mice (Voikar et al., 2004; Lindfors et al., 2006; Wanigasekara and Keast, 2006).

GFRα3 antiserum was raised against recombinant mouse GFRα3 extracellular domain (access No. AAB70931). The immunogen consisted of amino acid residues Glu34-Arg379 of the GFRα3 extracellular domain (manufacturer's technical information). This antibody recognizes the recombinant protein using a western blot. In direct ELISA, it shows approximately 10% cross-reactivity with recombinant human GFRα3 and less than 2% cross-reactivity with recombinant mouse GFRα2 and recombinant mouse GFRα4 (manufacturer's technical information). The GFRα3 antiserum has previously been used to identify neurons response to ARTN (Elitt et al., 2006; Malin et al., 2006).

2.6 CELL SIZE DISTRIBUTION

NIH ImageJ was used to measure the area of neurons. DRG sections were labeled with antibodies to PGP9.5 (a pan-neuronal marker), GFRα1, GFRα2 or GFRα3 separately. DRG from three animals were analyzed and 200 positively-stained neurons for each marker from each animal were measured.

2.7 ANALYSIS OF IN VITRO CELL SURVIVAL

To determine the amount of cell death over time in culture and the effect of growth factors on survival, neurons were plated on gridded, numbered coverslips. Eight squares from each coverslip were randomly selected and the number of neurons in each square was counted at 6, 24 and 96 h after plating (**Fig 6**). Neurons were grown in standard media with one of the following: NGF (50ng/ml), GDNF (50ng/ml), NRTN (50ng/ml), ARTN (20ng/ml) or without growth factor. Coverslips from six mice were analyzed for each condition. The cell number at 6 h in each condition was normalized as 100%. Data were analyzed using SigmaStat software. Significance was tested using a two-way ANOVA, and Dunnett's posthoc test.

2.8 RNA-ISOLATION AND REAL-TIME RT PCR

RNeasy Mini kits (Qiagen) were used to isolate total mRNA. RNA (1µg) was DNased (Invitrogen) to remove genomic DNA, and then reverse-transcribed using Superscript II reverse transcriptase (RT) (Invitrogen). Real-time RT PCR was performed as described previously (Malin et al., 2006) to determine the extent of expression of growth factor receptors in sensory neurons after sciatic nerve lesion. Primers optimized for real-time RT PCR were designed using ABI software (Molecular Biology Insights) and were shown in **Table 6**. Statistical significance was determined by t-test.

2.9 CALCIUM IMAGING

Calcium imaging was performed 16-20 h after culture. Cells were loaded with Ca^{2+} indicator by incubation in HBSS containing 5 mg/ml bovine serum albumin and 2 µM of the acetoxymethyl ester of fura-2 (Invitrogen) for 30 min at 37°C. Coverslips were placed on an Olympus microscope stage mount with 30°C HBSS buffer flowing at 5 ml/min. Firmly attached cells with IB_4-488 labeling were chosen and identified as regions of interest in the software (Simple PCI; C-Imaging, Compix Imaging Systems). Absorbance data at 340 and 380 nm were collected at 1 Hz, and the change in the 340/380 ratio analyzed. Ca^{2+} transients were examined in response to brief application of 1 µM capsaicin (Sigma) delivered onto neurons using a multi-barrel drug delivery system. To examine the response to menthol, we applied 250µM menthol (Sigma) followed by 1µM capsaicin.

2.10 IN SITU HYBRIDIZATION

The protocol for GFRα2 ISH was reported previously (Chen et al., 2006). Briefly, an ISH probe for GFRα2 was amplified using a nested RT-PCR strategy with gene-specific sets of PCR primers and cDNA templates prepared from P0 mouse whole brain. The probe was labeled with digoxigenin (Roche). To test the dependence of GFRα2 expression on the presence of functional Runx1, P30 *Runx1*$^{F/F}$ and *Runx1*$^{F/F}$*;Wnt1-cre* mice were perfused with 4% paraformaldehyde. Lumbar DRG were dissected, post-fixed for 1-2 h, cryoprotected overnight in 20% sucrose and cut at a thickness of 12 µm. Slides containing tissue sections with both genotypes were pretreated with proteinase K and TEA/acetic anhydride before being incubated overnight at 64°C with the GFRα2 probe. Reaction with the NBT/BCIP substrate (Roche) was allowed to proceed overnight. For experiments in which Runx1 ISH was combined with ATF3 immunolabeling, L3-5 DRG were collected 5 d after sciatic axotomy (see above). Ganglia were first stained with anti- antibody (rabbit 1:1000, Santa Cruz) and photographed, followed by development for Runx1 ISH using fluorescently-tagged nucleotides (Chen et al., 2006; Liu et al., 2008)

2.11 WESTERN BLOTTING

Pooled L2-4 DRG from WT (*n*=4) and NRTN-OE (*n*=4) mice were lysed in Trizol reagent (Invitrogen) and proteins were isolated following the manufacturer's instructions. For western blotting, protein concentration was determined using the Pierce 660 nm Protein Assay (Thermo Scientific). Aliquots containing 30 µg of proteins were dissolved in 0.1% SDS and boiled in 5×SDS loading buffer for 10 min, separated by 8% SDS-PAGE gel, transferred to PVDF

membranes, blocked, and incubated with primary antibodies at 4°C overnight. Primary antibodies used were rat anti TRPM8 (1:1000, a generous gift from Masatoshi Takeichi (Suzuki et al., 2007)) and rabbit-anti-GAPDH (1:5000, Santa Cruz Biotech). Horseradish peroxidase-coupled secondary antibodies were used for amplification and antibody binding visualized using a chemiluminescent detection kit (Thermo Scientific).

2.12 ESTIMATION OF NEURON NUMBER

The number of L4 DRG neurons was estimated as previously described (Harrison et al., 2004; Elitt et al., 2006). WT (n = 4) and NRTN-OE (n = 4) mice were given an overdose of avertin anesthetic followed by transcardial perfusion with 4°C saline, and the L4 DRG were collected. DRG were post-fixed in 4% paraformaldehyde for 30 min and then transferred to 25% sucrose. Serial sections with hematoxylin and eosin staining were analyzed. Sections were examined at a total magnification of 400× and a drawing tube was used to record neurons containing one or more nucleoli. Six evenly spaced sections were analyzed per ganglion. The number of neurons counted was multiplied by the interval between analyzed sections. These raw counts were then adjusted using a correction factor to account for the possibility that individual neurons had multiple nucleoli and could theoretically be counted more than once. The correction factor is the percentage of neurons with only one nucleolus. This correction factor was then multiplied by the raw count to provide an adjusted estimate of cell number. Statistical significance was determined by t-test.

2.13 NERVE HISTOLOGY

The saphenous nerves from WT (*n*=4) and NRTN-OE (*n*=4) mice were exposed at mid-thigh level, fixed with 4% paraformaldehyde, 2% glutaradehyde in 0.1 M PB for 10 min and a 3 mm segment was removed and post-fixed for 2 h, washed in 0.1M PB. Nerve segments were then dehydrated in graded alcohols, infiltrated with propylene oxide and embedded in epoxy resin and polymerized at 60°C for 24 h. Ultrathin sections (0.7-0.8 nm) were cut on an ultramicrotome, stained with lead citrate and uranyl acetate and photographed on an electron microscope. The numbers of myelinated and unmyelinated axons in each nerve cross-section were counted. The diameter of myelinated and unmyelinated axons was analyzed using NIH ImageJ. Statistical significance was determined by t-test.

2.14 INFLAMMATION

An emulsion of 50% complete Freund's adjuvant (CFA) was prepared by thoroughly mixed equal volumes of sterile saline with CFA. For behavioral tests, 10 μl of 50% CFA was injected subcutanously in the plantar surface of hindpaws.

2.15 THERMAL SENSITIVITY TESTING

Thermal sensitivity was assessed using the Hargreaves test. WT (*n*=10) and NRTN-OE (*n*=10) mice were placed in individual plexiglass chambers on a glass plate maintained at 30ºC.

Animals were acclimated for 1 h before testing. Response latencies (flinching or lifting the paw) to noxious thermal stimulation were measured by applying a radiant heat stimulus (15% intensity setting; IITC Inc.) to each hindpaw. The testing was performed using repeated measures (three measures per foot) of the left and right glabrous hindpaw skin. Six response latencies were averaged for each animal. Data were shown as mean ± SEM. Statistical significance was determined by t-test. In all behavioral experiments, the experimenters were blinded to the genotype of the mice.

2.16 MECHANICAL SENSITIVITY TESTING

Mechanical sensitivity was assessed using the von Frey filament test. Foot withdrawal frequencies in response to von Frey stimuli were performed as previously described (Schwartz et al., 2008). WT (*n*=8) and NRTN-OE (*n*=8) mice were placed in individual plexiglass chambers on a mesh screen platform and were acclimated for at least 10 min before testing. Mechanical stimuli were applied from below to the plantar surface of the right hindpaw. Mechanical sensitivity was determined by assessing the foot withdrawal responses in response to the von Frey monofilaments 3.61 (vF#3.61), which is equivalent to 0.4g force. Each test was composed of 10 stimuli for each area. The number of positive responses was converted into a percentage with 10 positive responses corresponding to 100%. Data were shown as mean ± SEM. Statistical significance was determined by t-test.

2.17 COLD PLATE TEST

WT (*n*=20) and NRTN-OE (*n*=20) mice were placed in a plexiglass container with an ice floor. The ice blocks were placed on top of dry ice and temperature the ice blocks were maintained at -20ºC and the latency to the first nocifensive response (foot lifting or jumping) and the number of responses in 60 sec was quantified. Statistical significance was determined by t-test.

2.18 THERMAL GRADIENT TEST

WT (*n*=20) and NRTN-OE (*n*=20) mice were placed into thermal gradient apparatus (IITC). Mice were allowed to explore 11 keys with temperature ranging from 16°C to 45°C for 60 mins. Mice were acclimated to the testing environment for 30 mins and the time the spent on each key from 30-60 mins were recorded and the percentage of time on each key will be calculated. Statistical significance was determined by two-way ANOVA.

2.19 TWO-TEMPERATURE CHOICE TEST

WT (*n*=20) and NRTN-OE (*n*=20) mice were placed into thermal gradient apparatus (IITC). Mice were allowed to explore two adjacent keys, with one held at 32°C and the other ranging from 4°C to 50°C (**Fig. 7**). Mice were acclimated to the testing environment for 20 min, and the time spent on each key during a 5 min interval were recorded. The percentage of time

spent on the 32°C surface was measured. Data were shown as mean ± SEM. Statistical significance was determined by two-way ANOVA and Bonferroni post-hoc test.

2.20 DRINKING BEHAVIOR

Mice were tested for oral sensitivity to menthol using a modified paired-preference drinking aversion paradigm as previously described(Elitt et al., 2008). Mice were housed in cages individually and given food and water *ad libitum*. Each cage was fitted with two bottles. Mice were allowed to drink freely from the two bottles for 24 h and then the volume consumed in each bottle was measured. For two days, both bottles contained normal water. On the third day, mice were tested for oral sensitivity for menthol. One bottle contained normal water plus vehicle (0.07% ethanol) and another bottle contained menthol at a concentration of 0.1, 1 or 5μM. Six WT and 6 NRTN-OE mice were used for each dose. Within group differences between water with vehicle and water with menthol were determined by two-way ANOVA and Bonferroni post-hoc test.

2.21 *EX VIVO* PREPARATION

An *ex vivo* somatosensory preparation in which the skin, nerve, DRG, and spinal cord are intact was used as previously described (Woodbury et al., 2001). Briefly, mice were anesthetized with an intramuscular injection of a mix of ketamine (90mg/kg) and xylazine (10 mg/kg). Animals were perfused transcardially with chilled (12-15ºC) oxygenated (95% O_2/5% CO_2) artificial CSF (aCSF; in mM: 253.9 sucrose, 1.9 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26.0 $NaHCO_3$ and

10.0 D-glucose). The spinal cord and the right hindlimb were excised and placed in a circulating bath of aCSF. In this circulating bath, the spinal cord, DRG, saphenous nerve, and the skin innervated by this nerve (hairy skin of the right hindpaw) were isolated. After dissection, the isolated preparation was transferred to a recording chamber containing oxygenated aCSF in which the sucrose had been replaced with 127.0 mM NaCl and the skin was pinned out on a mesh-covered platform. The level of the bath was lowered so that the dermal surface remained perfused with the aCSF while the epidermis was allowed to dry.

2.22 RECORDING AND STIMULATION

Somata in L2 and L3 DRG were impaled using quartz microelectrodes (impedance > 150 MΩ) containing 5% neurobiotin (Vector Laboratories) in 1M potassium acetate. The peripheral receptive field (RF) of the recording neuron was localized with a blunted glass stylus and von Frey hairs. When cells had no mechanical RF, a thermal search was conducted by applying hot (~52 ºC) and/or cold (~0ºC) physiological saline to the skin. The peripheral response properties of the DRG neurons were assessed using digitally controlled thermal and mechanical stimuli. Mechanical stimuli including a series of single 5 s square waves of varied forces (1, 5, 10, 25, 50, and 100 mN) were applied to the cell's RF using a 1-mm-diameter plastic disc. After mechanical stimulation, the response of the cell to thermal stimulation was determined using a 3 mm^2 contact area peltier stimulator (Yale University Machine Shop). The RF of the cells was slowly heated (15 s) from 31 to 52°C and held for 5 s before returning to 31°C in 12 s.

2.23 IMMUNOSTAINING OF RECORDED CELLS

After a sensory neuron was characterized and intracellularly filled with Neurobiotin, the DRG containing the injected cell was removed and processed for immunostaining as previous described (Jankowski et al., 2010). The following antibodies are used: Rabbit anti-TRPV1 (1:500; Calbiochem), goat anti-GFRα2 (1:500 R&D Biosystems), rabbit anti-CGRP (1:1000 Chemicon) and IB_4-488.

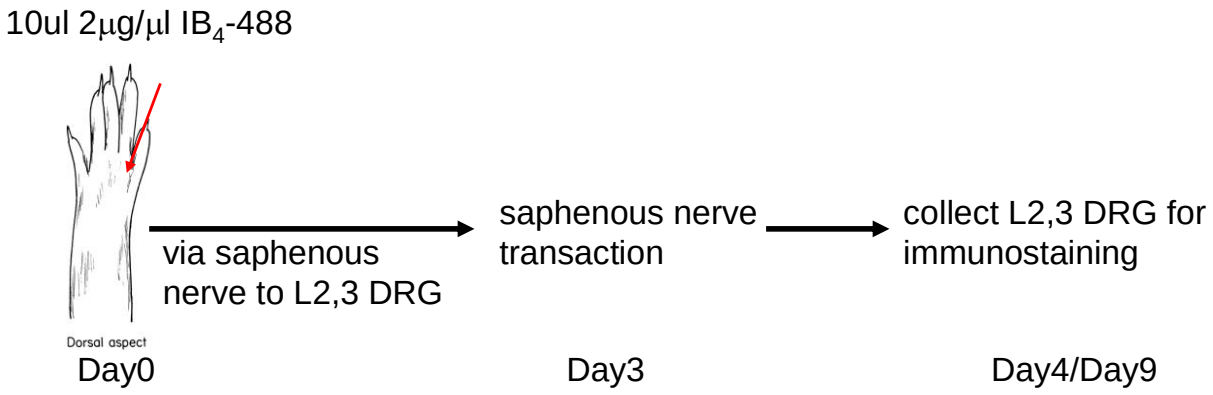


Figure 5. Diagram of IB_4-488 injection and saphenous nerve transaction time points.

IB_4-488 was injected into dorsal-medial part of hindpaw. Saphenous nerve transaction was performed on both sides 3 d after IB_4 injection. 1d and 6d after saphenous nerve transaction, L2, 3 DRG from both sides were collected for immunostaining. 6d after saphenous nerve transaction, L2, 3 DRG were collected and dissociated in dish for calcium imaging.

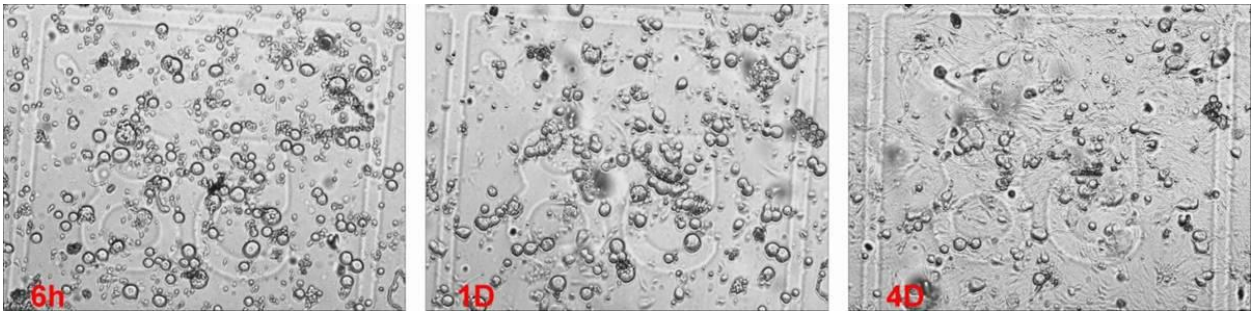


Figure 6. Method for neuronal cell survival assay.

DRG cells were plated on gridded, numbered coverslips, each square has an identical number. For example, the number for the square shown here is "58". The pictures of same square were taken at 6h, 1d and 4d after plating and number of neurons was counted.

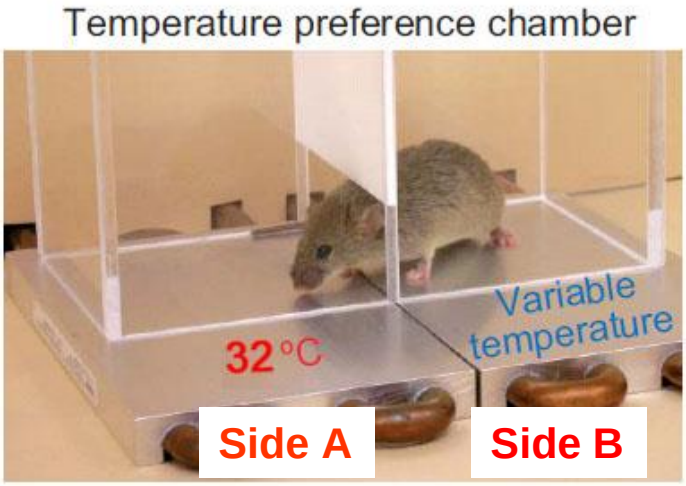


Figure 7. Mouse in temperature preference chamber for two-temperature choice test.

The temperature on side A is fixed to 32°C and the temperature on side B is different in each test, ranging from 4 to 50 °C. For each test, mouse was allowed to explore on the chamber for 5 mins and the time spent on each side were recorded. Modified from Bautista et al., 2007.

3.0 PHEOTYPIC SWITCHING OF NONPEPTIDERGIC CUTANEOUS SENSORY NEURONS FOLLOWING PERIPHERAL NERVE INJURY

3.1 ABSTRACT

In adult animals, the phenotype of half of all nociceptive sensory neurons is tonically modulated by growth factors in the GDNF family that includes GDNF, ARTN and NRTN. Each family member binds a distinct GFRα family receptor, such that GDNF, NRTN and ARTN bind GFRα1, -α2, and -α3, respectively. Previous studies reveal significant transcriptional regulation of all three receptors in response to axotomy, possibly in response to changes in growth factor availability. Here, we examined changes in the expression of GFRα1-3 in response to injury *in vivo* and *in vitro*. We found that after dissociation of adult sensory ganglia, up to 27% of neurons die within 4 d in culture and this can be prevented by NGF, GDNF and ARTN, but not NRTN. Moreover, upregulation of ATF3 (a marker of neuronal injury) *in vitro* could be prevented by NGF and ARTN, but not GDNF or NRTN. The lack of NRTN efficacy was correlated with rapid and near-complete loss of GFRα2 immunoreactivity. By retrogradely-labeling cutaneous afferents *in vivo* prior to nerve cut, we demonstrated that GFRα2-positive neurons switch phenotype following injury and begin to express GFRα3 and the capsaicin receptor, TRPV1. This switch was correlated with down-regulation of Runx1, a transcription factor that controls expression of GFRα2 and TRPV1 during development. These studies show

that NRTN-responsive neurons are unique with respect to their adult plasticity and response to injury, and suggest that Runx1 plays an ongoing modulatory role in the adult.

3.2 RESULTS

3.2.1 Distribution of GFRα1-3-expressing neurons in vivo and in vitro

Initial experiments were designed to determine the proportion of neurons expressing GFRα1-3 protein in situ (i.e. tissue sections of intact DRG) and in dissociated neurons (i.e., cultured) to elucidate the extent to which the phenotype of cultured neurons was representative of the *in vivo* condition. Using recently validated GFRα1-3 antibodies (Elitt et al., 2006; Kalous et al., 2007) we performed immunohistochemical staining on L4 DRG. TRPV1 immunoreactivity was also examined because it is expressed in 95% of GFRα3 neurons, but in only a minority of the IB_4-expressing population (Orozco et al., 2001). IB_4-labeling was used because of its extensive colocalization with GFRα1 and α2 (Bennett et al., 1998). A large number of cells immunoreactive for GFRα3 and TRPV1 were observed in L4 DRG of naïve mice (**Fig. 8*G, H, I***), whereas IB_4 was primarily localized in GFRα2- (**Fig. 8*M,N,O***) and small-diameter GFRα1-positive neurons (**Fig. 8*J,K,L***), confirming previous studies (Bennett et al., 1998; Baudet et al., 2000; Bennett et al., 2000) .

Figure 9 shows the size distribution of GFRα1-, GFRα2- and GFRα3-positive somata in L4 DRG. The size distribution of each GFRα population was interesting; GFRα1 staining was

seen in neurons with both the largest and smallest somata. Most GFRα2-positive neurons had somata with areas between 100-200 μm^2, whereas the majority of GFRα3-positive neurons had somata with areas below 150 μm^2.

Table 2 shows the percentage of mouse neurons that express GFRα1-, GFRα2- or GFRα3-immunoreactivity *in vivo* (i.e., in tissue sections from intact DRG) and 24 h after plating (the time point used typically for acute physiological analysis of dissociated sensory neurons). In intact ganglia, approximately 33% of all NeuN-positive (used to identify neurons) cells expressed GFRα1-immunostaining, whereas 33% expressed GFRα2 and 25% expressed GFRα3, similar to what has been reported in rat and mouse (Bennett et al., 2000; Orozco et al., 2001). After 24 h in culture the percentages were approximately 15%, 2% and 44% for GFRα1, GFRα2, GFRα3, respectively. The decrease in both GFRα1- and α2-positive cells were statistically significant, as was the increase in GFRα3 ($p<0.05$; T-test).

3.2.2 Extent of cell death in vitro and effects of growth factors

To determine whether the decrease of GFRα1 and GFRα2 expression was due to selective loss of specific cell populations after dissociation, we counted the number of neurons at 6 h (the time point at which all the cells that survive the dissociation process have attached to the coverslip), 1 d and 4 d after plating. Cells were grown on coverslips containing a numbered grid so that the same cells could be followed throughout the experiment. As shown in **Table 4**, without growth factor, 85.31% and 73.22% of neurons survived at 1 d and 4 d after plating, respectively. The minimal loss of cells seen at 24 h indicated that the decrease in the percent of cells expressing GFRα1 or GFRα2 was not due to selective cell death and was more likely due to downregulation

of receptor expression. Application of NGF, GDNF or ARTN significantly increased neuronal survival at both 1 and 4 d, whereas addition of NRTN did not prevent cell loss (Two way-ANOVA, df = (4 , 2), F =3.51, *p<0.05;, Dunnett's post-hoc test, compare with control group).

3.2.3 Expression of ATF3 is regulated by NGF and ARTN but not NRTN

Because cell death did not appear to account for the dramatic loss in the percent of GFRα2-expressing neurons, we examined whether there were overt differences in the pattern of gene expression in different populations following dissociation. ATF3 is a transcription factor that has been shown previously to be expressed in the vast majority of axotomized afferents, is thought to be a major regulator of the regeneration program (Seijffers et al., 2007), and to be under the control of growth factors ((Wang et al., 2003; Averill et al., 2004) (but see (Dussor et al., 2003)). To test how growth factors regulate ATF3 expression in different primary afferent populations after injury *in vitro,* we applied NGF (50 ng/ml), ARTN (20 ng/ml) or NRTN (50 ng/ml) at day 0 (The dose of each growth factors is same as previous work in our laboratory (Malin et al., 2006), which indicated that growth factors have physiological potentiation in sensory neuron response to capsaicin). Twenty-four hours after plating, 92% of DRG neurons (identified via NeuN expression) expressed ATF3-immunoreactivity and this level of staining was observed for up to 7 d in culture. To identify the specific effects of NGF, cells were stained with an antibody to CGRP, which has been shown previously to be expressed in 96% of neurons expressing TrkA (the specific NGF receptor) (Averill et al., 1995). Addition of NGF to the culture media eliminated ATF3 expression in 96% of CGRP-immunoreactive neurons at 1 and 4 d after culture (**Fig. 10**). Similarly, addition of ARTN to the culture media eliminated ATF3 expression in 91% of GFRα3-immunoreactive neurons both at 1 and 4 d in culture (**Fig. 11**).

Six hours after plating, GFRα2 immunoreactivity could still be detected. At this time ATF3 expression is seen in virtually all GFRα2-expressing neurons (**Fig. 12*A*-*C***). However, GFRα2 expression was dramatically decreased after 1 d in culture and virtually absent by day 4 (**Fig. 12*D*-*I***). Because both GFRα1- and GFRα2- positive cells decreased *in vitro* (**Table 2**), we examined the effect of NRTN or GDNF on all sensory neurons using NeuN-staining. NRTN or GDNF application had no effect on the percentage of cells expressing ATF3; 92% of all NeuN-positive cells expressed ATF3 in the presence of either growth factor. This was true even at 6 h after plating when over 95% of all neurons expressed ATF3 in the presence or absence of GDNF or NRTN.

We repeated these studies using IB_4 labeling because IB_4 labels both GFRα1 and α2 populations. Furthermore, in cultured neurons from mice that lack GFRα2, the percentage of IB_4 cells is identical to that in cultures established from WT mice (Stucky et al., 2002), indicating that IB_4-binding is independent of GFRα expression. Addition of NRTN, GDNF, or a combination of NRTN and GDNF was not able to suppress the expression of AFT3 in IB_4-binding neurons; 84.6% of IB_4-positive neurons expressed after 1 d in culture and this number was unchanged following addition of NRTN or GDNF (86.9% and 86.4%, respectively)(**Table 3**)(Two-way ANOVA, df = (3, 1), $F = 1.08$, $p = 0.48$). At 4 d in culture 78% of IB_4-positive cells expressed ATF3-immunoreactivity, whereas 77.9% and 79.0% expressed ATF3-immunoreactivity despite the addition of NRTN or GDNF. Addition of both growth factors also had no effect on expression at 1 d and 4 d (86% and 80% of IB_4-positive neurons expressed ATF3 at 1 and 4 d, respectively, after addition of both GDNF and NRTN)(**Table 3**)(Two-way ANOVA, df = (3, 1), $F = 1.08$, $p = 0.48$).

3.2.4 In vivo, axotomized GFRα2-expressing neurons downregulated GFRα2 and expressed GFRα3

Previous studies in rat indicated a near doubling in the number of DRG neurons expressing mRNA for GFRα1 and GFRα3 following axotomy *in vivo,* such that over 66% of sciatic afferents expressed GFRα1 and 66% expressed GFRα3 by day 14 post-nerve cut (Bennett et al., 2000). This high level of distribution of these two GFRα receptors indicates that some cells switched GFRα expression. The observed down-regulation in GFRα2 protein reported here and previously makes these cells likely candidates for switching receptor phenotype. To examine the phenotype of GFRα2-positive neurons after peripheral nerve injury *in vivo*, IB_4-488, which is selectively taken up by IB_4-binding neurons, was injected subcutaneously into the dorsal medial portion of the hindpaw prior to nerve transection. IB_4-488 can be visualized within back-labeled neurons for at least two weeks following injection. Because of the site of injection, IB_4-488 is retrogradely transported through the saphenous nerve to cutaneous afferent somata in the L2-3 DRG. IB_4-488 injected into the skin labels GFRα1 and GFRα2-expressing neurons (Bennett et al., 1998), but since the saphenous nerve is cutaneous, the majority of cells express GFRα2 (Lu et al., 2001). Three days after injection, IB_4-488 labeled a large number of neurons expressing GFRα2 (**Fig. 13 *A*-*D***). No cells expressing GFRα3 were co-labeled with IB_4-488 (**Fig. 13 *E*-*H***). In contrast, one day following saphenous nerve axotomy (4 d after IB_4-488 skin injection), ATF3 immunoreactivity was seen in IB_4-labeled neurons (**Fig. 13 *I*-*P***). By this time, the GFRα2 expression had already decreased to such an extent that it was difficult to find neurons that were GFRα2-positive and ATF3-positive, or alternatively GFRα2-positive cells that were back-labeled with IB_4-488 (indicating that they were axotomized) (**Fig. 13 *I*-*L***). By

day 6 post-axotomy 38.92 ± 2.81 % of IB_4-488-backlabeled neurons expressed GFRα3 (n=4) (**Fig. 13** ***U-X***) indicating that a significant proportion of neurons that previously expressed GFRα1 or GFRα2 now expressed GFRα3.

To determine whether saphenous nerve axotomy have effect on retrograde transport of IB_4-488, we compared the percentage of neurons that labeled by IB_4-488 in two groups of mice. Group one mice had IB_4-488 injection on d0 and L2, 3 DRG were collected at day 9 after injection. Group two mice had IB_4-488 injection on d0, saphenous nerve axotomy on d3 and L2, 3 DRG were collected at day 9 after injection. The percentage of neurons that were labeled by IB_4-488 has no significant difference between two groups, suggesting saphenous nerve axotomy had no effect on IB_4-488 transport during the test time points.

3.2.5 Injured cutaneous afferents upregulate TRPV1

As shown in **Figure 13**, most IB_4-488 back-labeled neurons from naïve mice express GFRα2. Only 27.3 ± 0.9 % (n=4) of these GFRα2-positive neurons express TRPV1 (**Table 5**). After saphenous nerve axotomy our data indicate that 39% of IB_4-488 back-labeled neurons downregulate GFRα1 or α2 and switch phenotype to express GFRα3. Since most GFRα3-positive neurons express TRPV1 (88.9 ± 0.9 %; n=4), we performed Ca^{2+} imaging and measured the response of IB_4-488 back-labeled neurons to capsaicin in naïve and axotomized mice. In naïve mice (n=3), 26.5 ± 4.3% of IB_4-488 back-labeled neurons responded to capsaicin (similar to previous reports (Breese et al., 2005)). However at 6 d after saphenous nerve axotomy (n=3), this percentage increased significantly (68.5 ± 7.5% (p<0.05; two way ANOVA)). No change occurred in the percentage of TRPV1 responders that were not back-labeled with IB_4-488

(capsaicin responses in naïve IB_4-488 -negative neurons = 43.6 ± 5.5%: capsaicin responses in 6 d axotomized IB_4-488 -negative neurons = 45.1 ± 0.3%) (**Fig. 14**). These data suggest that IB_4-488 back-labeled neurons acquire TRPV1 function following injury.

3.2.6 Changes in GFRα2 and Runx1 mRNA after axotomy

As noted above, previous studies in rat DRG showed changes in mRNA expression for GFRα1-3 14 d following sciatic nerve transection (Bennett et al., 2000). We confirmed these changes in mouse at 6 d following sciatic nerve transection using real-time RT PCR (**Fig. 15*B***). Transection of the saphenous nerve had similar effects but only reached significance for down-regulation of GFRα2 at day 1. This may reflect the fact that a smaller portion of the cells in L2-3 DRG contribute to the saphenous nerve and therefore changes in this population are masked by GFRα2 expression levels in uninjured cells.

During development, Runx1 expression is required for the proper differentiation of Ret-expressing neurons (including those co-expressing GFRα2) from the TrkA-positive population, which contains the precursors for the majority of nociceptive neurons (Kramer et al., 2006; Luo et al., 2007; Yoshikawa et al., 2007). In the adult, Runx1 is expressed in the majority of Ret/IB_4-positive neurons including 92% of the Mrgprd-positive neurons (a G-protein coupled receptor found in 75% of IB_4-positive neurons (Zylka et al., 2005)), 79% of TRPM8 expressing neurons and 15% of CGRP-expressing neurons (Abdel Samad et al., ; Chen et al., 2006). In Runx1 KO mice, no cells are lost but the number of Ret-expressing neurons is decreased by over 50%. However, the percentage of IB_4-positive neurons is normal, indicating that not all characteristics of the non-peptidergic afferent population are regulated by Runx1 (Chen et al., 2006). **Figure**

15A shows that in Runx1 KO mice GFRα2 expression is greatly decreased, suggesting that GFRα2 is also under control of Runx1 expression. That Runx1 continues to control GFRα2 expression is suggested by the observation that following axotomy, the decrease in Runx1 correlates with a decrease in GFRα2, but not GFRα1 or GFRα3 (**Fig. 15*B***). ISH confirmed that axotomized neurons, produced by sciatic nerve lesion and visualized by ATF3 staining, exhibited no Runx1 signal 5 d post-axotomy (**Fig. 15*C***), although Runx1 mRNA could be visualized in ATF3-negative neurons. These data suggest Runx1 expression is required to maintain GFRα2 expression in the adult.

3.3 DISCUSSION

The results presented here confirm previous studies showing that sensory neuron populations defined by expression of GFRα1-3 respond differently following *in vivo* axotomy or dissociation *in vitro* (a procedure that includes peripheral and central axotomy). The present experiments extend those studies by demonstrating that the GFRα2 population is unique in that following injury *in vivo* and *in vitro*, these neurons rapidly downregulate expression of GFRα2, and begin to express a phenotype typical of the GFRα3 population, including expression of functional TRPV1 receptors. Moreover, *in vitro* phenotypic changes associated with injury can be prevented in some sensory populations by growth factors including NGF and ARTN, whereas the changes seen in the GFRα2 population cannot be reversed by addition of NRTN. Interestingly, the loss of GFRα2 may be regulated by the transcription factor Runx1, as Runx1 is

required for the developmental expression of GFRα2 and Runx1 is downregulated *in vivo* following nerve transection.

3.3.1 Survival effect of GFLs

In many ways, growth factors in the GDNF family have unique and complementary roles to those in the neurotrophin family (NGF, BDNF, NT-3 or NT-4/5). Although there is a small population of cells during early development that express Ret (these appear to become primarily low threshold mechanoreceptors (Luo et al., 2007; Luo et al., 2009)), the majority of GFL-responsive neurons initially express TrkA during early development and only begin to express Ret and GFRα receptors after the period of embryonic programmed cell death (Molliver et al., 1997; Luo et al., 2007). Transgenic ablation of NGF, BDNF or NT-3 produces a significant reduction in the number of sensory neurons present in adult DRG and trigeminal ganglia. In contrast, genetic deletion of GFL or their receptors has a more mild effect on DRG and trigeminal neurons, probably due to the fact that expression of GFRα 1-3 and Ret is initiated in most neurons later in development (Valdes-Sanchez et al., ; Heuckeroth et al., 1998; Mendell et al., 1999; Baloh et al., 2000a; Honma et al., 2002), after the period of programmed cell death when up to half of all embryonic sensory neurons undergo apoptosis (Davies and Lumsden, 1984). *In vitro*, we observed a small (but significant) increase in neuronal survival of adult neurons upon addition of NGF, GDNF or ARTN but no effect for NRTN. These effects were expected given the phenotype of GFL or GFRα KO mice. However, GFLs have been reported to significantly increase the survival of embryonic and neonatal neurons (Baudet et al., 2000). The more dramatic survival effect of GFL on young sensory neurons *in vitro* versus that seen in

KO mice may reflect the response of young neurons to the injury sustained during dissociation; i.e., the process of culturing young neurons may initiate death programs that can be reversed by addition of growth factors. It should be noted that neuronal survival in the absence of growth factors is low for embryonic and neonatal sensory neurons (<20% for neurons < P15), whereas survival for adult neurons is > 70% 4 d after culturing ((Baudet et al., 2000), current results), again suggesting that neonatal neurons are more vulnerable to injury-induced cell death. Indeed, recent studies showed that BDNF is required for survival of neonatal nociceptors *in vivo* (Valdes-Sanchez et al.). The observation that NRTN had no effect on the survival of adult neurons probably reflects the fact that GFRα2 is almost completely absent in cultured neurons within 24 h.

3.3.2 Regulation of ATF3 expression by growth factors in vitro

ATF3 was uniformly upregulated in all neurons examined regardless of the changes in GFRα receptor expression *in vivo* or *in vitro*. This suggests the importance of ATF3 to regeneration (Seijffers et al., 2007). GFRα2 is expressed in the majority of cutaneous afferents (ca. 70%; (Lu et al., 2001; Lindfors et al., 2006)) and these correspond physiologically to polymodal C-fibers (CPM) (Koerber and Woodbury, 2002; Woodbury et al., 2004). These fibers appear to regenerate at the same rate as other C-fibers (Jankowski et al., 2009) and thus, expression of GFRα2 does not appear to be a prerequisite for regeneration.

NRTN appears to share its lack of effectiveness with GDNF in terms of not being able to block expression of ATF3 *in vitro*. Again, this could be due to the loss of GFRα2 expression, however GFRα2 is still present at 6h, a time at which ATF3 is expressed in virtually all neurons

in vitro. Thus, in culture only NGF and ARTN can block upregulation of ATF3. Interestingly, in mouse, 80% of neurons that express GFRα3 also express TrkA (Orozco et al., 2001) so it is not clear if the ability to regulate ATF3 is specific to the population of cells expressing these two receptors or if downstream signaling is different when Ret interacts with GFRα3 compared to Ret activation in combination with GFRα1 and GFRα2. (However it should be noted that a population of GFRα3-expressing neurons may not express Ret (Bennett et al., 2006)). That this is not the case is suggested by the studies of Averill et al (Averill et al., 2004) that showed *in vivo* that both NGF and GDNF could prevent the upregulation of ATF3 in adult rat when applied intrathecally for two weeks. These results indicate that long-term activation of Ret via GDNF can regulate ATF3. Why this does not happen *in vitro* during the short time course examined here could be due to numerous differences in the experimental paradigm including species, the effect of culturing and the time course of the two experiments.

3.3.3 GFRα2 population is unique in response following injury

24 hours after dissociation in dish, the expression of GFRα2 had decreased to the extent that can not be detected by immunostaining. Application of NRTN or GDNF or both could not reverse the loss of GFRα2. Whether other growth factors such as NGF can prevent the decrease of GFRα2 after injury is still not clear. Previous work showed that overexpression of NGF in skin increased the size of IB_4–positive neurons in DRG, suggesting that NGF affects the development of GFRα2-positive neurons (Goodness et al., 1997). Expression of GFRα2 in DRG reduced dramatically in NGF KO mice (Luo et al., 2007), suggesting that NGF is required for the expression of GFRα2 in early development. So in the future, it will be of interest to investigate

whether application of NGF could reverse the loss of GFRα2 expression after dissociation of DRG neurons.

Previous studies by Stucky and others indicated that GFRα2 expression was required for detection of noxious heat in dissociated IB_4-binding neurons (Stucky et al., 2002). Because only 27% of GFRα2-positive neurons express TRPV1 (**Table 5**) and these neurons have normal heat sensitivity in TRPV1 KO mice (Woodbury et al., 2004), they possess the ability to transduce heat stimuli independently of TRPV1. However, following axotomy, the data presented here indicate that some of these neurons begin to express TRPV1 as early as 6 d after saphenous nerve transaction. Jankowski et al. (2009) did not detect an increase of TRPV1 mRNA expression in DRG until 4 weeks after saphenous nerve injury. The difference between the two studies is probably because we investigated the functional change of TRPV1 in single cell level. Six day after axotomy, the TRPV1-responded neurons increased from 26% to 68% in IB_4-488 back-labeled neurons. However, the IB_4-488 back-labeled neurons only present a small percentage of neurons in L2,3 DRG, so the change the TRPV1 may not be able to be detected by Real-time RT-PCR analysis using mRNA from all L2,3 DRG neurons. The switch of phenotype could theoretically explain the increased thermal sensitivity of CPMs to noxious thermal stimulation seen after regeneration (Jankowski et al., 2009). That IB_4-positive neurons are capable of expressing TRPV1 in culture following inflammation has been reported (Breese et al., 2005), although other laboratories have seen no change in cultured inflamed cutaneous afferents(Lu et al.). Thus, the ability of inflammation to induce *de novo* TRPV1 expression in cutaneous afferents remains controversial.

3.3.4 Correlation of Runx1 and GFRα2 expression after nerve injury

GFRα2 is not the only sensory neuron growth factor receptor that is decreased following injury; both p75 and TrkA are somewhat decreased following peripheral nerve lesion, whereas both TrkB and TrkC have been reported to increase (Verge et al., 1992; Ernfors et al., 1993). However, the decrease observed in TrkA and p75 can be blocked by exogenous NGF (Verge et al., 1992), whereas NRTN has no effect on GFRα2 expression. Thus GFRα2-expressing neurons may be hard-wired to downregulate the expression of this receptor and to lose the ability to respond to NRTN. One possible mechanism for this difference is that GFRα2 may continue in adulthood to be dependent on the expression of Runx1, which regulates its expression during development. Thus, when Runx1 is decreased following axotomy, GFRα2 is also decreased. During embryogenesis, TrkA is co-expressed with Runx1 and appears to be regulated by Runx1 (Chen et al., 2006; Kramer et al., 2006; Marmigere et al., 2006; Yoshikawa et al., 2007). However, during late development Runx1 is downregulated in TrkA-expressing neurons, presumably freeing TrkA from regulation by this transcription factor. The observed up-regulation of GFRα1 and GFRα3 indicate that either these receptors are not regulated by Runx1 in the adult or that Runx1 is acting as a repressor, an action it exerts during development ((Bennett et al., 2000; Marmigere and Ernfors, 2007), present results).

3.4 CONCLUSION

In summary, the studies conducted here indicate that GFRα2-expressing neurons are unique relative to other populations of sensory afferents identified based on growth factor receptor expression. GFRα2 is rapidly and dramatically downregulated in response to injury *in vitro* and *in vivo* and these neurons appear to switch phenotype so that they can respond to a related growth factor (ARTN) and express TRPV1, a channel not normally seen in these neurons. These changes have obvious functional implications for the development of cutaneous hypersensitivity following injury. Identification of the signaling changes that lead to these alterations could provide new and useful therapeutic targets.

	GFRα1	GFRα2	GFRα3	TRPV1	IB4
***in vivo* (%)**	33.5 ± 1.9	33.3 ± 2.6	24.8 ± 0.8	30.3 ± 1.5	28.3 ± 0.8
***in vitro* (%)(24h)**	15.8 ± 1.0*	2.1 ± 0.6%*	43.9 ± 2.9*	36.3 ± 0.6*	43.6 ± 1.4*

Table 2. The percentage of DRG neurons that express GFRα1-, GFRα2-, GFRα3- and TRPV1- immunoreactivity *in vivo* and 24 h after culturing.

GFRα2-positive cells are rare 24 h after plating. The percentage of GFRα1- positive cells is decreased whereas GFRα3-positive cells are increased. An increase in the percentage of TRPV1-positive and IB_4-binding neurons also occurs (* indicate significant change; $p<0.05$, t-test).

	Control	NRTN	GDNF	GDNF+NRTN
1d (%)	84.6 ±1.4	86.9 ±1.4	86.4 ± 2.5	86.0 ± 2.1
4d (%)	78.2 ±1.5	77.9 ±1.6	79.0 ± 2.3	80.0 ± 1.6

Table 3. Application of NRTN, GDNF or both can not suppress ATF3 expression in IB_4-binding neurons.

One day and 4 d after culturing, cells were stained with IB_4 and ATF3. The percentage of IB_4 neurons that express ATF3 were shown in table. Compare with control, application of NRTN, GDNF or both to the medium did not decrease the ATF3 expression in IB_4 –positive neurons. Two-way ANOVA, df = (3, 1), $F = 1.08$, $p = 0.48$.

	Control	NGF	GDNF	Artemin	NRTN
6h (%)	100	100	100	100	100
1d (%)	85.31±0.69	96.24±1.13*	92.38±2.06*	94.12±2.09*	81.95±5.23
4d (%)	73.22±3.27	89.53±1.64*	87.71±1.80*	88.48±1.94*	76.16±4.43

Table 4. Sensory neuron survival after 1 d or 4 d in culture.

The number of NeuN-positive neurons at 6 h post plating was designated as 100%. Without growth factors (Control), approximately 15% of neurons died at 1 d after plating and 27% died by 4 d. Application of NGF, GDNF and ARTN significantly increased neuron survival although NRTN was ineffective. Two way-ANOVA, df = (4 , 2), F =3.51, *p<0.05;, Dunnett's post-hoc test, compare with control group.

% TRPV1 that are GFRα1+	24.1± 0.8
% TRPV1 that are GFRα2+	21.0 ± 0.5
% TRPV1 that are GFRα3+	79.8 ± 1.4
% GFRα1 that are TRPV1 +	23.1 ± 0.6
% GFRα2 that are TRPV1 +	27.3 ± 0.9
% GFRα3 that are TRPV1 +	88.9 ± 0.9
% IB_4 that are GFRα1+	65.2 ± 1.3
% IB_4 that are GFRα2+	89.2 ± 0.9
% IB_4 that are GFRα3+	6.9 ± 0.3
% GFRα1 that are IB_4+	53.3 ±1.5
% GFRα2 that are IB_4+	74.0 ± 0.1
% GFRα3 that are IB_4+	8.7 ± 0.6

Table 5. Overlap between GFRα1, 2, 3 and IB4, TRPV1.

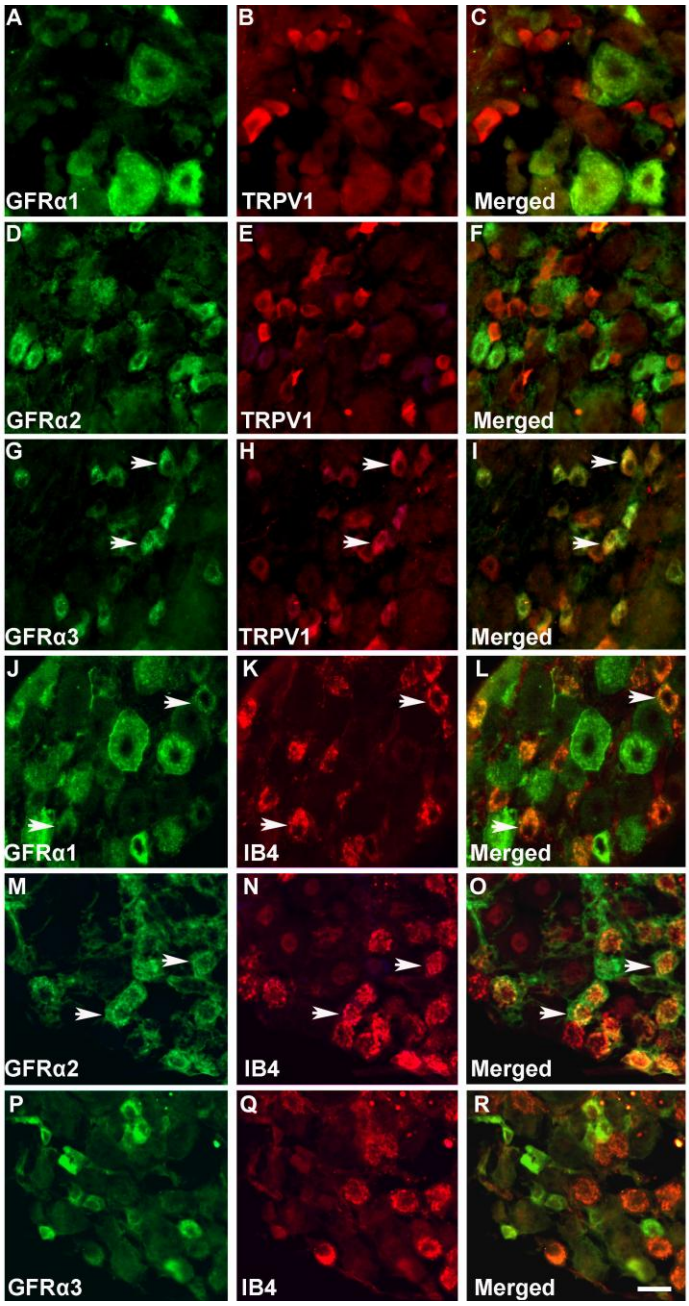


Figure 8. Immunolabeling for GFRα1, 2 or 3 and TRPV1 and IB_4 in L4 DRG.

Most GFRα1 and α2 neurons did not express TRPV1 (***A-F***). GFRα3-positive neurons expressed TRPV1 (***G-I***). GFRα2 (***M-O***) and small diameter GFRα1-positive neurons (***J-L***) bound IB_4. Scale bar=50 μm.

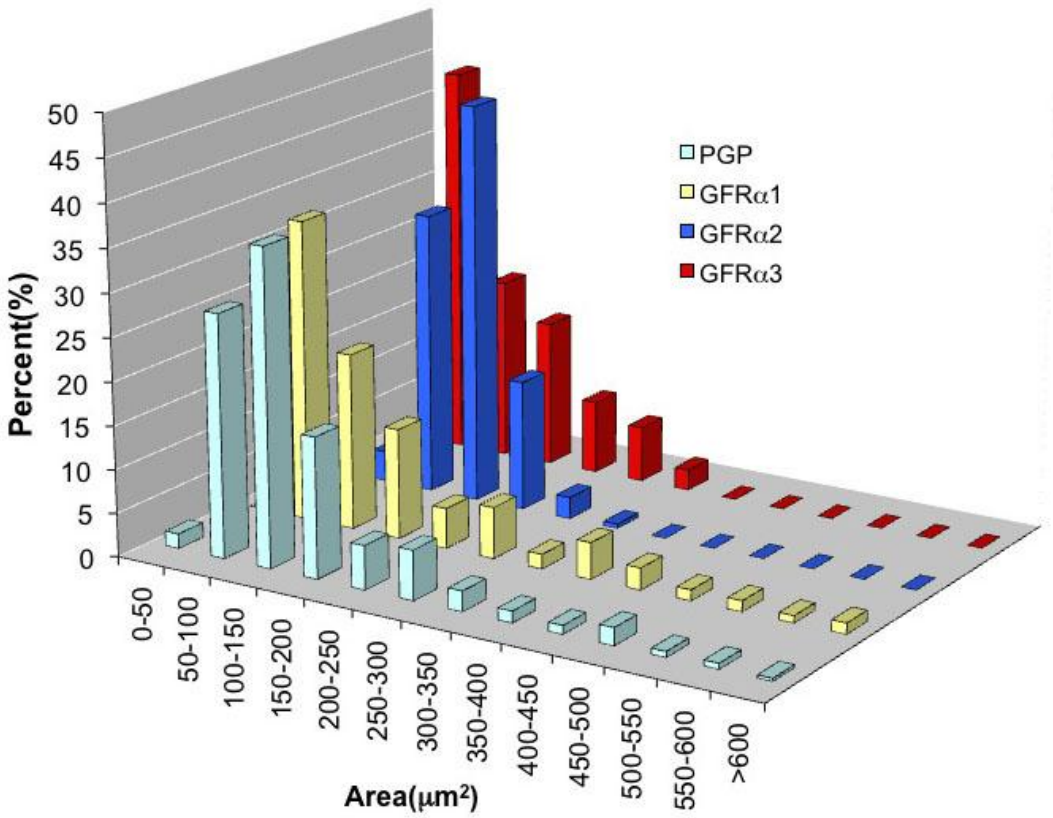


Figure 9. Cell size distribution of PGP9.5-, GFRα1-, GFRα2- and GFRα3-positive neurons in L4-5 DRGs.

Neurons were sorted by their cell area and the percent of neurons within each 50 μm bin were plotted. PGP9.5-staining was used to obtain a distribution for all neurons. Note that GFRα1-immunoreactivity was expressed in both small and large sized neurons. Most GFRα2-positive neurons had areas between 100-250 μm^2 whereas many GFRα3-positive neurons were classified as small neurons.

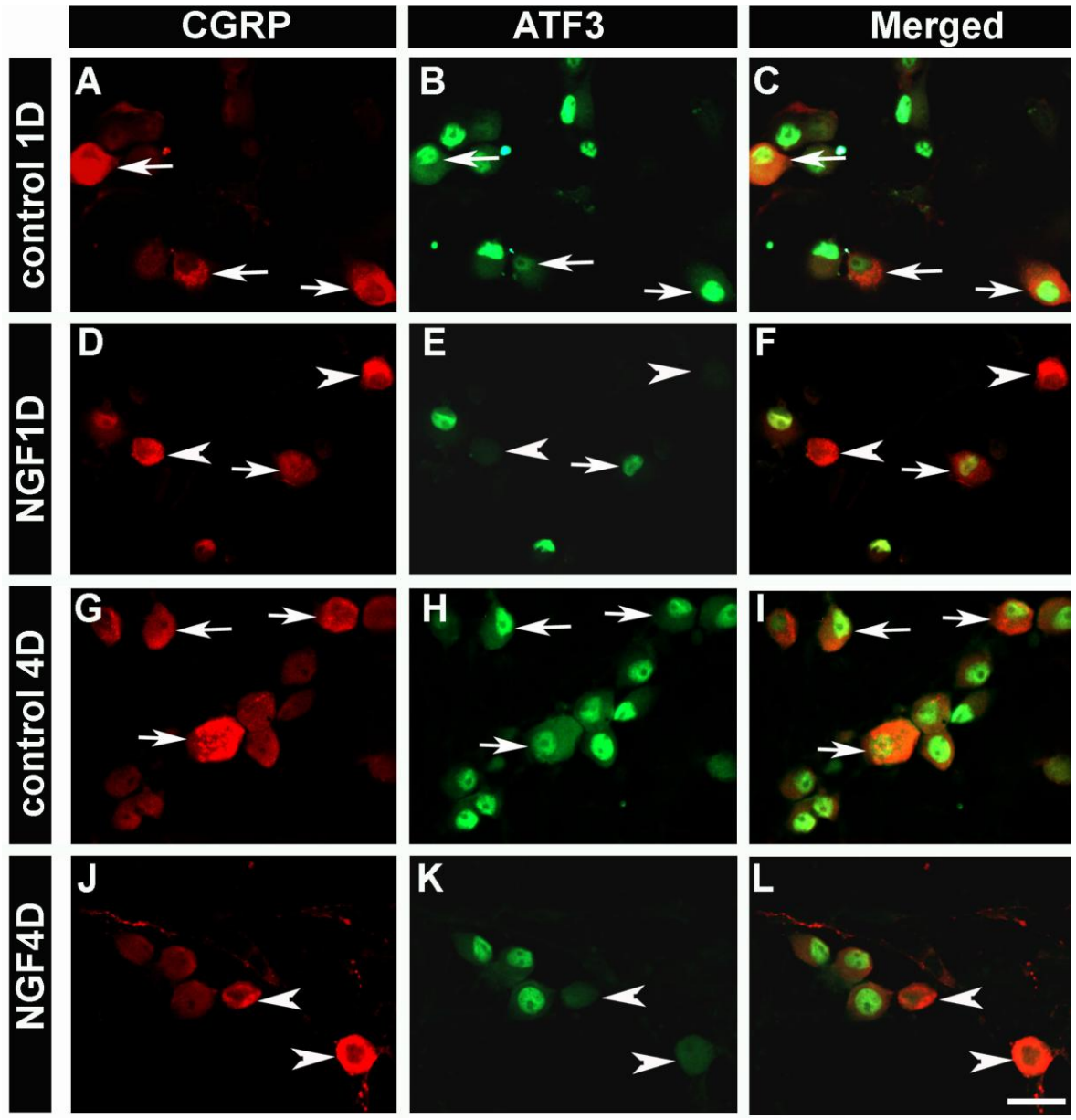


Figure 10. *In vitro* application of NGF decreased ATF3 expression in CGRP-positive neurons.

In control conditions (no growth factor), virtually all CGRP-positive neurons expressed ATF3 at 1 d (***A-C*** arrows) and 4 d (***G-I*** arrows) after plating. In NGF treated cultures, CGRP-positive neurons exhibited a decrease (96%) in ATF3 at 1 d (***D-F*** arrowheads) and 4 d (***J-L*** arrowheads). Scale bar =50μm.

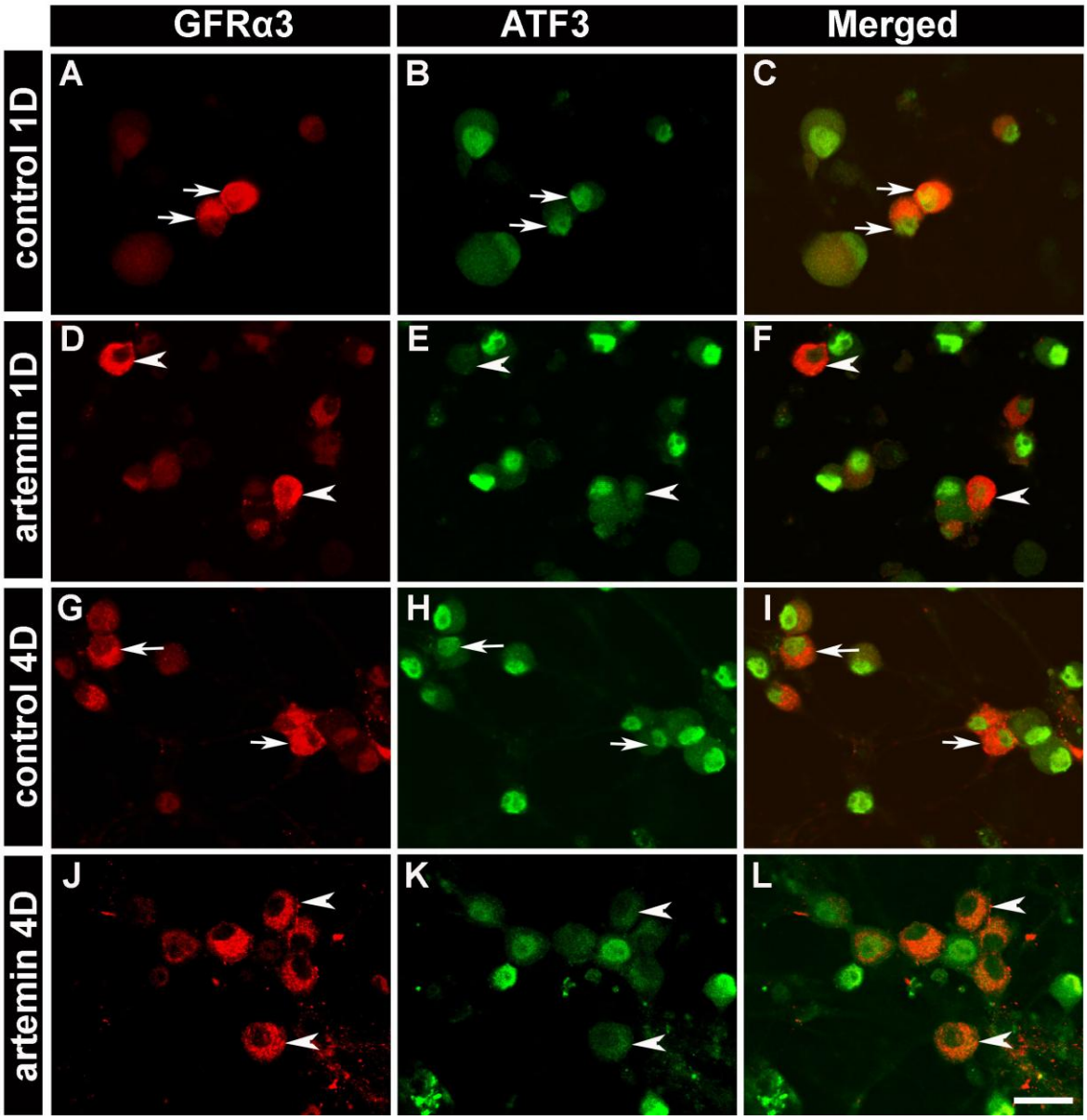


Figure 11. ***In vitro*** **application of ARTN decreased ATF3 expression in GFRα3-positive neurons.**

In control conditions, all GFRα3-positive neurons expressed ATF3 at 1 d (***A-C*** arrows) and 4 d (***G-I*** arrows) after plating. Addition of ARTN decreased the percentage of ATF3-positive GFRα3-staining neurons by 91% at 1 d (***D-F*** arrowheads) and 4 d (***J-L*** arrowheads). Scale bar=50 μm.

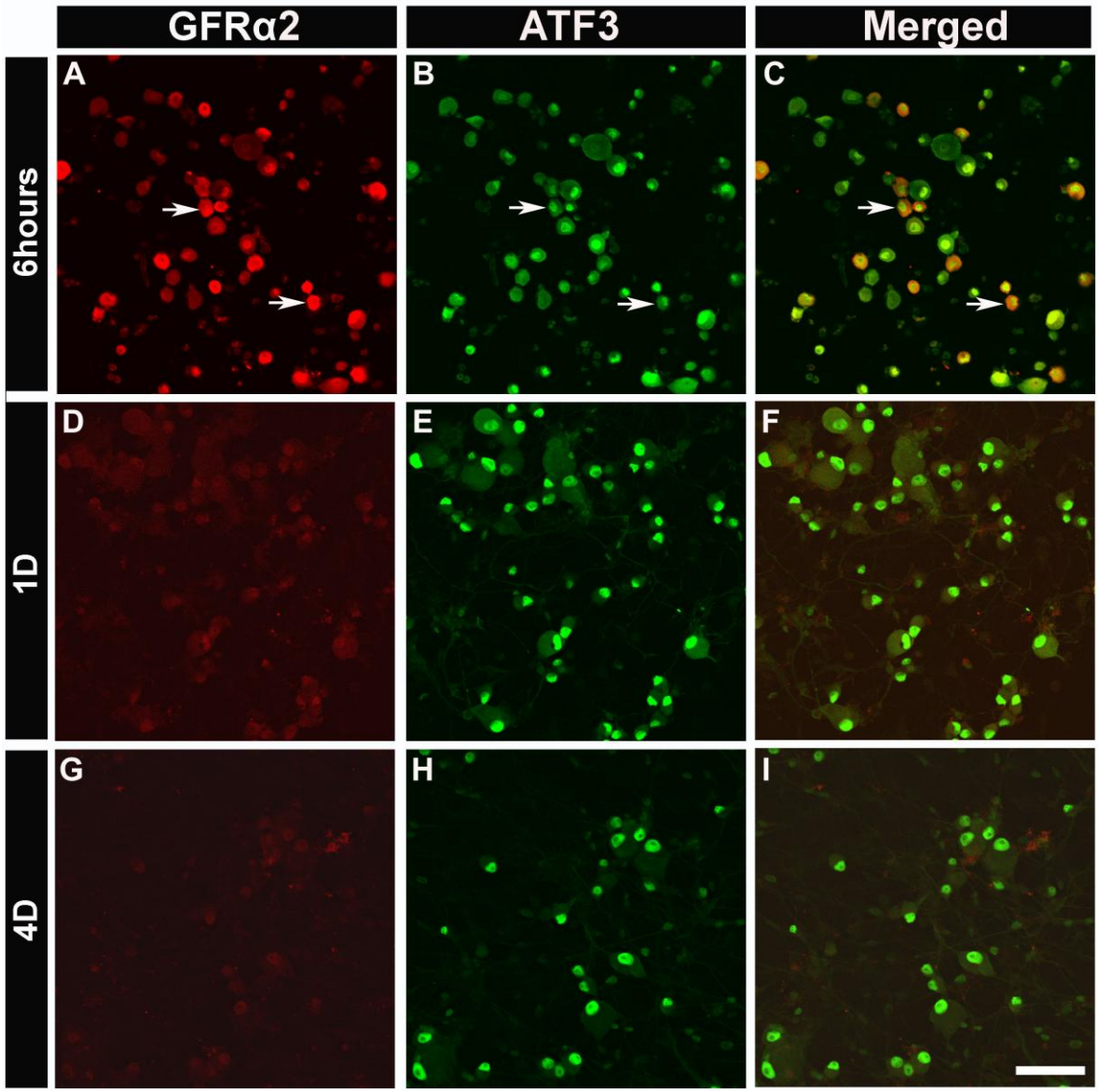


Figure 12. The percentage of GFRα2-positive neurons decreased after plating and this can not be reversed by application of NRTN or GDNF.

Six hours after plating, 32% of neurons expressed GFRα2 (***A,*** arrow) and most GFRα2-positive neurons expressed ATF3 (***B, C,*** arrows). One day and 4 d after plating the expression of GFRα2 was not detected (***D, F***). Scale bar=100 μm

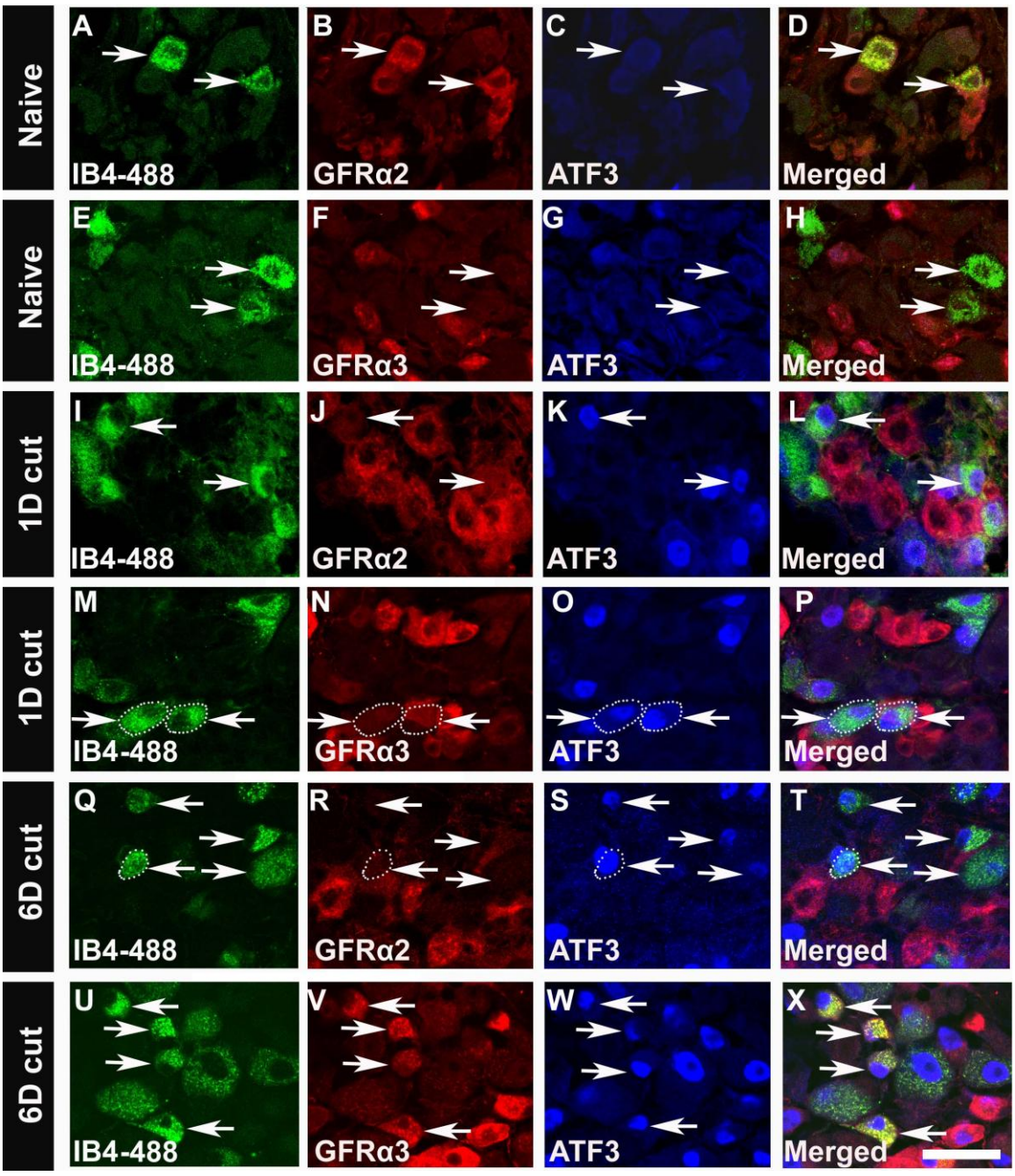

Naive
A
IB4-488
B
GFRα2
C
ATF3
D
Merged
Naive
E
IB4-488
F
GFRα3
G
ATF3
H
Merged
1D cut
I
IB4-488
J
GFRα2
K
ATF3
L
Merged
1D cut
M
IB4-488
N
GFRα3
O
ATF3
P
Merged
6D cut
Q
IB4-488
R
GFRα2
S
ATF3
T
Merged
6D cut
U
IB4-488
V
GFRα3
W
ATF3
X
Merged

Figure 13. After saphenous nerve injury GFRα2-positive neurons downregulate GFRα2 and express GFRα3.

In naïve mice, IB_4-488-labeled neurons were immunoreactive for GFRα2 (***A, B, D;*** arrows), but not GFRα3 (***E, F, H;*** arrows). No ATF3 expression was seen following IB_4-488 injections in naïve mice (***C, G;*** arrows). One day after saphenous nerve lesion, IB_4-488-labeled neurons expressed ATF3 (***I, K, M, O*** arrows), however, GFRα2 expression in IB_4-labeled neurons was decreased (***I, J;*** arrows). GFRα3 expression was not seen in IB_4-488-labeled neurons 1 d post-transection (***M, N, P;*** arrows). Six days after saphenous nerve lesion, IB4-488-labeled neurons expressed ATF3 (***Q, S, U, W*** arrows), GFRα3 (***U, V, X*** arrows) but not GFRα2 (***Q, R, T*** arrows). Scale bar=50 μm

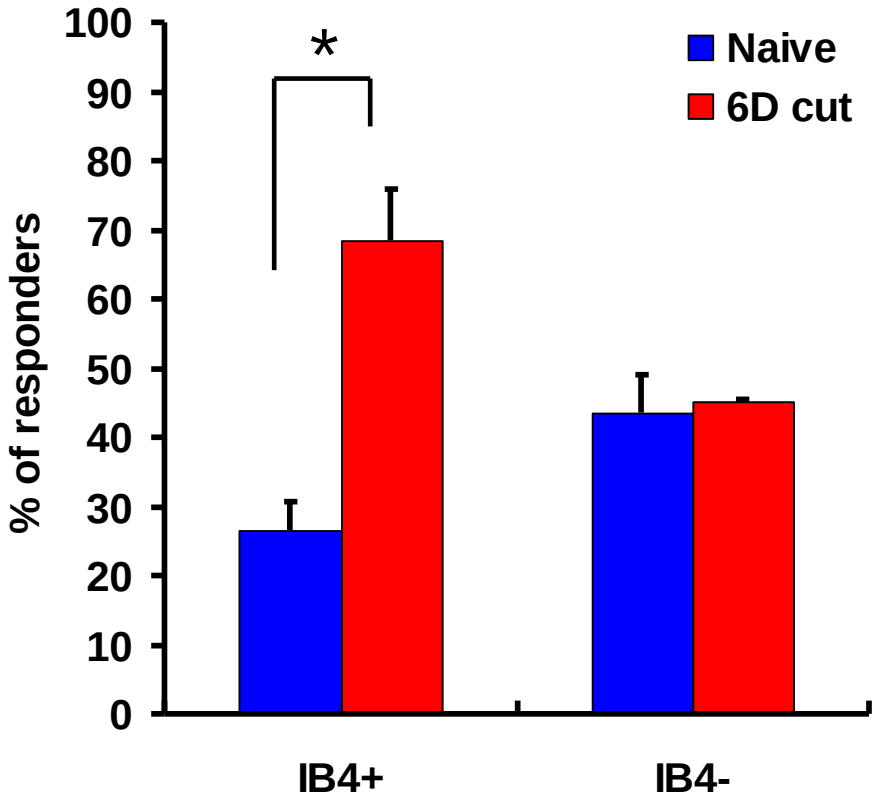
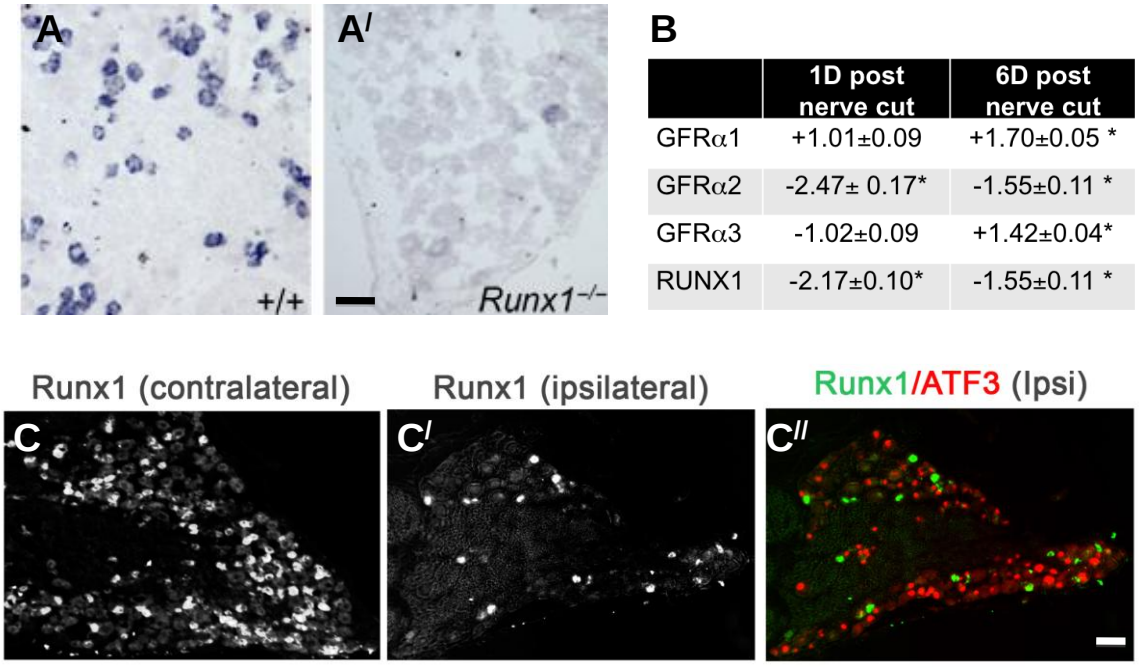


Figure 14. Six days after saphenous nerve transaction, the percentage of IB_4-488 back-labeled (IB4+) neurons that responded to capsaicin increased significantly.

In naïve mice (n=3), 26.5 ± 4.3% of IB_4-488 back-labeled neurons responded to capsaicin. At 6d after saphenous nerve transaction (n=3), this percentage increased significantly to 68.5 ± 7.5%. No change in the percentage of TRPV1 responders that were not labeled with IB_4-488 (IB4-). Fisher's exact test, *p<0.05.



	1D post nerve cut	6D post nerve cut
GFRα1	+1.01±0.09	+1.70±0.05 *
GFRα2	-2.47± 0.17*	-1.55±0.11 *
GFRα3	-1.02±0.09	+1.42±0.04*
RUNX1	-2.17±0.10*	-1.55±0.11 *

Figure 15. GFRα2 and Runx1 expression are decreased after nerve injury.

In situ hybridization for GFRα2 in WT (***A***) and Runx1 KO (***A′***) mouse DRG showed significant reduction of GFRα2 in Runx1 KO ganglia. Calibration bar = 50 µm. **B**) Fold change of GFRα1-3 and Runx1 mRNA following sciatic nerve transection. GFRα2 and Runx1 decreased concurrently. GFRα1 and α3 increased at 6 d post transection confirming previous rat ISH studies (Bennett et al., 2000). n=4,**p*<0.05; T-test. **C**) Five days after sciatic nerve axotomy, Runx1 expression decreased in ipsilateral DRG (***C′***) relative to contralateral (***C***) DRG. Runx1 expression (green) was absent in ATF3 positive neurons (red) in ipsilateral DRG(***C″***). Scale bar = 50 µm. In collaboration with Qiufu Ma's lab in Harvard University.

4.0 NEURTURIN OVEREXPRESSION IN SKIN ENHANCES EXPRESSION OF TRPM8 IN CUTANEOUS SENSORY NEURONS AND LEADS TO BEHAVIORAL SENSITIVITY TO COLD AND MENTHOL

4.1 ABSTRACT

NRTN, a member of the GDNF family of neurotrophins, binds the GPI-anchored protein GFRα2 and the receptor tyrosine kinase Ret. Expression of the GFRα2 receptor is primarily restricted to the peripheral nervous system. It is expressed in polymodal nociceptors that make up the majority of C-fiber innervation of the epidermis. These cells have primarily small somata and unmyelinated fibers that bind IB_4. To determine how NRTN affects sensory neuron properties, transgenic mice that overexpress NRTN in skin keratinocytes (NRTN-OE mice) were analyzed. Overexpression of NRTN in skin increased the density of PGP9.5-positive, but not CGRP-positive free nerve endings in footpad epidermis. GFRα2-positive neurons were hypertrophied in NRTN-OE mice and most of these neurons expressed the coolling-sensitive channel TRPM8. Expression of NRTN increased the level of mRNA encoding GFRα2, Ret, TRPM8, the ATP receptor $P2X_3$ and ASIC2a. Behavioral testing of NRTN-OE mice showed they had increased mechanical sensitivity, increased heat sensitivity and decreased sensitivity to noxious cold. NRTN overexpression also increased behavioral sensitivity during cold plate testing with temperatures at 17°C and 20°C and increased oral sensitivity to menthol. This

increase in cold and menthol sensitivity was found to correlate with increases in TRPM8 expression and the percentage of menthol-responsive cutaneous sensory neurons in NRTN-OE mice. These data indicate that enhanced expression of NRTN in the skin modulates gene expression and leads to behavioral sensitivity to thermal and mechanical stimuli.

4.2 ANATOMICAL RESULTS

4.2.1 Overexpression of NRTN induces epidermal hyperinnervation

The human epidermal K14 keratin promoter was linked to the mouse NRTN cDNA (**Fig. 16*A***) to direct overexpression of NRTN to basal keratinocytes of the epidermis. This promoter is activated from E11 through adulthood. The K14 promoter has been shown to successfully direct high-level expression of NGF, BDNF, NT3, GDNF and ARTN in the epidermis of skin (Albers et al., 1994; Albers et al., 1996; Zwick et al., 2002; Albers et al., 2006; Elitt et al., 2006). Real-time RT PCR analysis showed an enhancement of NRTN mRNA levels in the skin of NRTN-OE mice by 1123% in glabrous skin and 313% in flank skin (**Fig. 16*B*** and **Table 8**).

The increase of NRTN in skin induced hyperinnervation of PGP9.5-positive but not CGRP-positive fibers in the epidermis of hindpaw glabrous skin (**Fig. 17**). In previous studies, overexpression of NGF, NT-3 and GDNF by the K14 promoter led to an increase in the number of DRG neurons (Albers et al., 1994; Albers et al., 1996; Albers et al., 2006). However, a comparison of counts of the number of neurons in L4 DRG of WT (n=4) and NRTN-OE (n=4) mice showed no difference (WT, 3927± 62; NRTN-OE, 3930 ± 54). Confirming the lack of effect on cell number, counts of saphenous nerve axons also revealed no change (**Table 7**).

4.2.2 NRTN modulates expression of the GFRα2 receptor in TG and DRG

The canonical receptor complex for NRTN is composed of Ret receptor tyrosine kinase and GFRα2 (Heuckeroth et al., 1999; Rossi et al., 1999; Baloh et al., 2000a). To determine how increased availability of NRTN affected sensory neurons, immunostaining of GFRα2 in TG and L4 DRG was performed. The percentage of GFRα2-positive neurons was increased by approximately 30% in NRTN-OE DRG (WT, 33.3 ± 2.6; NRTN-OE, 43 ± 1.3; $p<0.01$, t-test). Cell area measures showed hypertrophy of GFRα2-expressing neurons in both TG (WT, 164.6 ± 3.0μm^2; NRTN-OE, 335.5 ± 7.6μm^2; $p<0.001$) (**Fig. 18 *A*-*F***) and L4 DRG (WT, 196.0 ± 4.5μm^2; NRTN-OE, 290.4 ± 8.2μm^2; $p<0.001$) (**Fig. 18 *G*-*L***). Real-time RT PCR analysis showed GFRα2 mRNA increased 62% in pooled lumbar DRG and 98% in TG (**Table 9**) in NRTN-OE mice. The Ret mRNA level also increased 77% in DRG and 144% in TG, whereas the TrkA mRNA level was unchanged (**Table 9**). Given that there was no increase in the number of DRG neurons, these data indicate that the increase in expression of GFRα2 and Ret occurred on a per cell basis. Whether the increase occurred because cells that normally expressed these molecules increased their level of expression or whether this represents *de novo* expression is not possible to determine with the present data.

4.2.3 NRTN increases the diameter of cutaneous nerve axons

Low magnification electron microscopic montages showed that the diameter of the saphenous nerve was larger in NRTN-OE mice (**Fig. 19 *A, B***). The number of myelinated and unmyelinated axons in saphenous nerve was not changed (**Table 7**). This is consistent with the

lack of change in the total neuron numbers in DRG. However, the mean diameter of both myelinated (WT: 3.64 ± 0.11μm; NRTN-OE: 3.92 ± 0.23μm; $p<0.05$) and unmyelinated (WT: 0.86 ± 0.04μm; NRTN-OE: 1.12 ± 0.05μm; $p<0.05$) axons increased significantly (**Table 7**). The axon diameter distribution histogram showed a uniform rightward shift for unmyelinated axons in NRTN-OE mice (**Fig. 19*C***). This is consistent with the hypertrophy of GFRα2-positive neurons observed in DRG. Most GFRα2-positive neurons are unmyelinated C-fibers (Snider and McMahon, 1998; Stucky and Lewin, 1999) and account for 70% of cutaneous afferents (Lu et al., 2001). These observations are also consistent with the observed increase in peripheral innervation; given the lack of increase in neuronal number, the increased number of epidermal fibers indicates terminal sprouting that would in turn lead to axonal and somatic hypertrophy to support increased metabolic demands of larger terminal projections.

Changes in myelinated axons were more subtle in NRTN-OE mice. The greatest effect was seen in the largest diameter fibers (5-6μm). This suggests that NRTN overexpression selectively affects a subset of myelinated cutaneous afferents, which are the early Ret and GFRα2-positive neurons that have large soma sizes (287 ± 102 μm^2) {Luo, 2009 #397}. 100% of early Ret-positive neurons also express GFRα2 and neurofilament 200. In contrast, almost none of early Ret-positive neurons express CGRP, nor did they bind IB_4. These neurons are rapid adapting mechanoreceptors, which form Meissner corpuscles, Pacinian corpuscles, and longitudinal lanceolate endings (Luo et al., 2009).

4.2.4 NRTN regulates the expression of receptors and TRP channels in DRG and TG

4.2.4.1 TRP channels

In preliminary studies performed using Illumina microarray gene chips on mRNA extracted from pooled L3-5 DRG. A significant number of sensory neuron-specific genes were found to change in NRTN-OE mice. Those genes with predicted functions were selected for validation using real-time RT PCR. The first gene that was examined was the thermosensitive gene TRPM8.

Real-time RT PCR analysis indicated the mRNA level of TRPM8 was significantly increased in both lumbar DRG (44%) and TG (84%) in NRTN-OE mice (**Table 9**). Immunolabeling of L4 DRG with antibodies to TRPM8 and IB_4-488 (**Fig. 20**) showed most IB_4-488-binding neurons expressed TRPM8 in NRTN-OE mice, whereas in WT mice, very few IB_4-488-positive neurons expressed detectable levels of TRPM8-immunoreactivity. Western blotting analysis also showed a dramatic increase of TRPM8 protein level in the DRG of NRTN-OE mice (**Fig. 21**).

Another putative temperature sensitive channel mRNA that was increased in NRTN-OE mice was TRPA1. It is generally agreed that thermal hyperalgesia induced by inflammation is decreased in TRPA1 KO mice (Kwan et al., 2006; Bautista et al., 2007), however its role in thermal detection is controversial. Some laboratories have provided evidence that it contributes to detection of noxious cold (Story et al., 2003; Elitt et al., 2006), whereas others have found no evidence for this role (Bautista et al., 2006).

4.2.4.2 Receptors

As shown in **Table 9,** NRTN overexpression also changed expression of various receptors in DRG and TG. We found an increase in the mRNA level of Ret and the ATP receptor $P2X_3$, both of which are expressed in most of GFRα2-positive neurons (Lindfors et al., 2006).

GluR5 is one of five subunits of the kainate receptors (one of the three families of ionotropic glutamate receptors) (Chittajallu et al., 1999). In neonatal rat DRG, 90% of kainate-responsive neurons bind IB_4 (Lee et al., 2001). GluR5 expression is significantly decreased in DRG and TG of NRTN-OE mice.

ASIC2 is a proton-gated sodium channel that is required for normal mechanosensitivity (Price et al., 2000). There are two splice variants of ASIC2: ASIC2a and ASIC2b (Lingueglia et al., 1997; Waldmann and Lazdunski, 1998; Garcia-Anoveros et al., 2001). Real-time RT PCR analysis showed an increase of ASIC2a mRNA in the DRG (95%) and TG (125%) of NRTN-OE mice (**Table 9**), whereas the mRNA level of ASIC2b was not changed.

Other receptors and channels that are expressed in peptidergic neurons, such as TrkA, p75, CGRP, and TRPV1, were not changed in NRTN-OE mice. Because these molecules are primarily expressed in peptidergic sensory neurons, their lack of change in NRTN-OE ganglion was predicted.

4.3 BEHAVIORAL ANALYSIS

4.3.1 NRTN overexpression increases oral sensitivity to menthol

We observed a significant increase of TRPM8 mRNA level in the TG (84%) (**Table 9**). TRPM8-expressing neurons in the TG have branches in lingual nerve fibers that innervated the tongue (Abe et al., 2005). To test whether NRTN-OE mice had increased oral sensitivity to menthol, a TRPM8 ligand, a two-bottle drinking aversion test was performed. For the first two days, mice were allowed to choose between two bottles of water. All mice drank the same volume of water from each bottle and did not display a bottle preference. On the third day, one bottle was filled with vehicle (0.14% ethanol in normal water), while the other was filled with menthol solutions of different concentrations. Neither WT nor NRTN-OE mice exhibited a bottle preference at the lowest concentration (0.1mM menthol). However, at higher concentrations (1.0 and 5.0 mM menthol), both WT and NRTN-OE drank less menthol water than normal water, and NRTN-OE mice consumed significantly less menthol water compare with WT (**Fig. 22**). This result suggested that NRTN-OE mice had increased oral sensitivity to menthol.

4.3.2 NRTN overexpression increases behavioral responses to cold stimuli

The change in neurochemistry noted above suggested that the function of channels associated with thermal sensitivity were altered in the NRTN-OE mice. TRPM8 is a channel protein reported to be activated by menthol or cold (Bautista et al., 2007). Since we found an increase in mRNA and protein for TRPM8, we performed a two-temperature choice test to determine whether NRTN-OE mice are sensitive to cold. In this test, WT (n=20) and NRTN-OE (n=20)

mice were allowed to explore two adjacent keys, with one held at 32°C and the other ranging from 4°C to 50°C. 32°C was chosen as the reference temperature because in preliminary experiments this was the preferred temperature for both WT and NRTN-OE mice when allowed a choice between 16°C and 45°C **(Fig. 23)** The percentage of time spent on each key was measured over a 5 min period. When the temperature of both keys was maintained at 32°C, mice spent equal time on each key, indicating no preference. When the temperature of one key was varied from 4°C to 50°C, both WT and NRTN-OE mice showed a significant preference for the key maintained at 32°C. Interestingly, when the comparison temperature was 17°C and 20°C, NRTN-OE mice spent significantly more time on the 32°C key, indicating that NRTN-OE mice were more sensitive to these temperatures (**Fig. 24**). When then comparison temperature was 41°C, NRTN-OE mice spent significantly less time on the 32°C key, indicating that NRTN-OE mice had decreased sensitivity to 41°C compared with WT (**Fig. 24**).

4.3.3 NRTN overexpression decreases the behavioral sensitivity to noxious cold stimuli

TRPA1 has been implicated in the detection of noxious cold (Story et al., 2003; Elitt et al., 2006). Because of the increase in TRPA1 mRNA in DRG and TG we investigated behavioral responses to noxious cold using a -20°C ice block. The number of nocifensive behaviors (jumping, foot lifting) that occurred in 60 s was measured. NRTN-OE mice had significantly longer response latency compared to WT mice (WT, 13.50 ± 0.97s; NRTN-OE, 21.74 ± 1.84s; n=20; p<0.001) (**Fig. 25**). The number of behaviors in 60s were also decreased in NRTN-OE mice (WT, 21.72 ± 1.97; NRTN-OE, 16.21 ± 1.76; n=20; p<0.05) (**Fig. 26**). Thus,

NRTN overexpression decreased sensitivity to noxious cold despite the increase in TRPA1 mRNA.

4.3.4 NRTN overexpression does not alter the behavioral sensitivity to noxious heat

NRTN-responsive neurons make up the majority of fibers innervating the epidermis and functionally, these afferents are primarily polymodal nociceptors responsive to mechanical and heat stimuli. Previous electrophysiological studies using dissociated DRG neurons from GFRα2 KO mice showed that NRTN/GFRα2 receptor signaling is required for noxious heat responses (Stucky et al., 2002). To test if changes in sensitivity to noxious heat occur after NRTN overexpression, we measured the hindpaw withdraw latency after exposure of footpads to radiant heat using a Hargreaves apparatus. Repeated measures showed the withdrawal latency were not different between WT and NRTN-OE mice (WT, 11.21 ± 0.50 s; NRTN-OE, 9.81 ± 0.45 s; *n*=20) (**Fig. 27**), indicating that overexpression of NRTN does not alter the sensitivity to noxious heat.

4.3.5 NRTN overexpression increases the behavioral response to mechanical stimuli

As noted in Section 4.2.2, NRTN-responsive neurons are dominated by polymodal nociceptors with the ability to detect mechanical stimuli of various intensities. We also found an increase of ASIC2a expression in DRG of NRTN-OE mice similar to that observed in GDNF-OE mice that was positively correlated with a change in mechanical sensitivity (Albers et al., 2006b). Therefore, we hypothesized that NRTN-OE mice may also display increased mechanical

sensitivity. Behavioral tests for mechanical sensitivity of WT and NRTN-OE mice were conducted by measuring hindpaw withdrawal frequencies in response to von Frey filament stimuli. Overexpression of NRTN significantly increased the mechanical sensitivity in naïve mice (WT, 20± 2.50%; NRTN-OE 42.5 ±3.85%, t-test, $p<0.05$) (**Fig. 28**).

4.4 CALCIUM IMAGING AND PHYSIOLOGY RESULTS

4.4.1 NRTN enhances the response to menthol in cutaneous sensory neurons

Overexpression of NRTN increased TRPM8 mRNA and protein in sensory neurons and increased behavioral response to cold and menthol stimuli, suggesting that NRTN enhances TRPM8 channel responses in sensory neurons. To further examine TRPM8 activity, we used calcium imaging to assess the response of WT and NRTN-OE cutaneous sensory neurons to the TRPM8 agonist menthol. IB_4-488 was injected subcutaneously into the medial-dorsal side of hindpaw to retrogradely label non-petidergic cutaneous afferents (primarily those expressing GFRα2) (as shown in **Fig.13**). Pooled L2-4 DRG neurons cultured for 16-20 hours were exposed to 250 μM of menthol that was washed out and followed by 1 μM capsaicin. In WT mice (n=3), 14.02 ± 1.38% of IB_4-488 labeled neurons responded to menthol, whereas the percentage was significantly increased to 48.81 ± 1.68% in NRTN-OE mice (n=3) (**Table 9**). The magnitude of the menthol-evoked calcium transient was unchanged between WT (0.39 ± 0.13 ΔF) and NRTN-OE (0.35 ± 0.05 ΔF) neurons. As a control to test the specificity of this change, we applied 1 μM capsaicin to the same neurons after menthol. We found no difference in capasaicin response between IB_4-488 labeled DRG neurons from WT (30.22 ± 0.31%) and NRTN-OE (32.29

±1.47%) mice, suggesting that skin-derived NRTN modulates menthol sensitivity in a TRPM8-specific manner.

4.4.2 Overexpression of NRTN in skin increases conduction velocity of C-fibers

Because of the observed changes in axonal diameter, conduction velocity (CV) of A- and C-fibers was measured in DRG neurons of WT and NRTN-OE mice. The mean CV of A-fibers was not changed in NRTN-OE mice (WT: 13.48 ± 2.02 m/s; NRTN-OE: 13.92 ± 1.50 m/s), whereas the CV of C-fibers was significantly increased. As shown in **Table 11**, in NRTN-OE mice, CH, CM, CMH and CMCH fibers had increased CV compared to WT mice. This is consistent with the finding that the entire distribution of C-fiber diameters was shifted to the right in NRTN-OE mice.

4.4.3 Overexpression of NRTN in skin increases the percentage of CM fibers in DRG

As shown in **Figure 29**, the percentage of CM fibers is significantly increased from 11% in WT mice to 21% in NRTN-OE mice, whereas the percentage of other types of C-fibers (CC, CH, CMH, CMCH) is not changed. The increase in CM fibers combined with the lack in change in the percent of other fibers suggests that the additional CM fibers are being recruited from the population of "silent" C-fibers that have been estimated to account for up to 30% of all afferents (Koerber et al., ; Jankowski et al., 2009). Interestingly, the increase in CM is almost entirely due to the appearance of afferents with high (>100mN) mechanical thresholds (**Table 12**). One explanation for this is that afferents that were previously silent have been activated by excess NRTN, but just to the extent that they could be detected with intense mechanical stimuli.

4.4.4 Overexpression of NRTN in skin does not change heat sensitivity of C-fibers

Because detection of noxious heat was reduced in GFRα2 KO mice (Stucky et al., 2002), we predicted that overexpression of NRTN might produce an increase in heat sensitivity. To compare the heat sensitivity of C-fibers, the firing frequency per degree of heating (over the course of the 20 s heat ramp) was calculated (**Fig. 30**). There was no difference between WT and NRTN-OE mice. In addition, the heat thresholds of CM, CMH and CMCH were not different between WT and NRTN-OE mice (Table 13). These results indicate that NRTN overexpression did not alter heat responsiveness of individual afferents.

4.4.5 Overexpression of NRTN in skin decreased the cold threshold of CMCH fibers

Because TRPA1 mRNA increased 74% in DRG (**Table 9**), and TRPA1 can be activated by cold temperatures with an average activation threshold of 17°C, we predicted that NRTN-OE mice had increased cold sensitivity. We compared the cold threshold of CMCH fibers and found that NRTN-OE mice had significantly lower cold threshold (12.26 ± 1.19 °C) comparing with WT (16.17 ± 1.65 °C) (t-test, $p<0.05$) (**Fig. 31**).

4.5 DISCUSSION: ANATOMICAL EFFECTS OF NRTN OVEREXPRESSION

In this study, we show that NRTN induced hypertrophy of GFRα2-positive/IB_4-binding neurons and induced hyperinnervation of nonpeptidergic fibers in the skin. We also demonstrated that overexpression of NRTN modulated gene expression and behavioral sensitivity to thermal and mechanical stimuli.

4.5.1 NRTN does not regulate DRG sensory neuron survival

Our results indicate that transgene-driven overexpression of NRTN in skin and tongue keratinocytes increases the number of neurons that express specific phenotypic markers previously associated with NRTN-responsive afferents; i.e., GFRα2 expression and binding of IB_4. In addition, there was an increase in the level of TRPM8 expression and behavioral correlates (enhanced sensitivity to cool and menthol) that are likely due to this increased expression. Unlike other growth factors such as NGF, NT-3 or ARTN, overexpression of NRTN did not increase the total number of neurons in DRG (Albers et al., 1994; Albers et al., 1996; Albers et al., 2006b). However, the percentage of GFRα2-expressing neurons was significantly increased. Whether the increase of GFRα2-positive neurons is due to increased survival of GFRα2 neurons during development (with a concomitant and equal loss of an unidentified population) or upregulation of GFRα2 in another population of neurons is not clear. Our real-time RT PCR results showed a decrease in the mRNA level of GFRα3 in DRG and TG. This could result from some GFRα3-expressing neurons switching to GFRα2 in response to increase NTRN levels. It should be noted that during development there are some cells that express both

GFRα2 and α3 (Baudet et al., 2000). Thus, NRTN does not increase the survival of DRG sensory neurons. This is consistent with previous results in NRTN-deficient mice that showed the total neuron number is not changed, although the expression of GFRα2 is lost in DRG and TG (Heuckeroth et al., 1999).

4.5.2 NRTN overexpression alters anatomical properties of cutaneous afferents.

In addition to an increased number of GFRα2-positive neurons, the soma diameter of GFRα2-positive neurons also increased in the DRG of NRTN-OE mice. The GFRα2-positive/IB_4-binding neurons had increased branching and appeared to hyperinnervate the skin. The diameter of unmyelinated fibers in saphenous nerve also increased. These results were predicted based on previous findings in GFRα2 KO and NRTN KO mice. These mice exhibited a decrease in the size of IB_4-binding neuronal somata and the density of CGRP-negative free nerve endings in the footpad skin was markedly reduced (Lindfors et al., 2006). The axon diameter of saphenous nerve was also decreased in GFRα2 KO mice (Stucky et al., 2002). In NRTN KO mice, the size of GFRα2-expressing neurons was reduced significantly in DRG and TG (Heuckeroth et al., 1999). These results suggest that although NRTN is not necessary for survival, it regulates the size of peripheral projections and indirectly the size of the supporting axons and somata of NRTN-responsive neurons.

4.5.3 NRTN regulates of TRP channels and other receptors

One of the most striking findings of our anatomical characterization of the NRTN-OE mice was the robust increase observed in TRPM8 expression in DRG. Real-time RT PCR analysis showed an increase of TRPM8 mRNA in both TG and DRG. Western blotting showed TRPM8 protein level was increased in DRG. In WT DRG, TRPM8 is expressed in small-diameter neurons that do not bind IB_4. However in NRTN-OE DRG, besides the small-diameter, IB_4-negative neurons, most of the GFRα2-positive, IB_4-binding neurons have high intensity of TRPM8 expression.

How NRTN upregulates TRPM8 expression in GFRα2-positive neurons is still not clear. In WT mice, TRPM8 is expressed in 5-20% of adult DRG neurons. These neurons are small in diameter, and do not express TRPV1, CGRP or Ret or bind IB_4 (Peier et al., 2002; Chen et al., 2006). During development, expression of TRPM8 is first detected at E16.5 in mouse DRG. Like GFRα2, initiation of TRPM8 expression requires Runx1, as no TRPM8 expression can be detected in Runx1 KO mice (Chen et al., 2006). At E10.5-E11.5, most Runx1-positive neurons express TrkA, and TrkA is required for TRPM8 expression because TRPM8 expression is completely abolished in the ganglia of TrkA null mice (Peier et al., 2002). Thus, the same signaling controls the initiation of GFRα2 and TRPM8 expression in development. Runx1 also regulates the expression of other TRP channels and receptors such as TRPV1, TRPV2, TRPA1, TRPC3, $P2X_3$, Nav1.9, Mrgprd4 and Mrgprd5 (Chen et al., 2006). The expression of some of these channels/receptors, such as TRPA1 and $P2X_3$, is increased in NRTN-OE DRG and TG, suggesting that Runx1 function is upregulated in NRTN-OE mice. The K14 keratin promoter drives NRTN expression from E11 (see Section 4.2.1), and upregulation of Runx1 as a result of

enhanced NRTN expression may result in increased expression of TRPM8, TRPA1 and $P2X_3$. TRPV1 expression is also regulated by Runx1, but its expression is not increased in NRTN-OE mice, probably because Runx1 is only required for the neurons that expressed a high level of TRPV1, but not all TRPV1-positive neurons (Chen et al., 2006).

NRTN also increased expression of ASIC2a, which is a proton-gated sodium channel that is required for normal mechanosensitivity (Price et al., 2000). In mice DRG, most IB4-binding neurons expressed ASIC2. Overexpression of GDNF increased the expression of ASIC2 in IB4-positive neurons this is correlated with an increase in mechanical sensitivity (Albers et al., 2006a). Since both GDNF and NRTN had effect on same population of neurons which is the IB4 binding neurons in DRG, it is not surprised that the expression of ASIC2a is also increased in NRTN-OE mice DRG.

4.5.4 NRTN might regulate the size of receptor field of cutaneous DRG neurons

Previous studies using transgenic mice which had overexpression of growth factors such as NGF, GDNF, and ARTN indicated that each of these growth factors increased survival of a specific subpopulation of sensory neurons in DRG and induced hyperinnervation of neuronal fibers into footpad skin (Albers et al., 1994; Zwick et al., 2002; Elitt et al., 2006).

Overexpression of NGF in skin increased the survival of sensory neurons and induced hyperinnervation of skin (Albers et al., 1994). The additional processes in the skin of NGF-OE mice made functional synaptic contacts for at least one type of sensory neuron, the high-threshold mechanoreceptors. The NGF-OE mice had a significant lower mechanical threshold (Davis et al., 1993). Overexpression of GDNF increased the survival of small unmyelinated sensory neurons that express Ret and bind IB4 and induced hyperinnervation of PGP9.5-positive

fibers into footpad skin (Zwick et al., 2002). Overexpression of ARTN caused a 20.5% increase in DRG neuron number and increases the intensity of TRPV1 expression in epidermal afferents (Elitt et al., 2006). NRTN also increased the percentage of GFRα2-positive neurons in DRG and induced hyperinnervation of PGP9.5-positive fibers into footpad skin. However, unlike other growth factors, NRTN increased the peripheral innervation without increasing the total number of DRG neurons. NRTN increased the diameter of GFRα2-positive neurons and these neurons had more branching into their peripheral targets. We did not measure the receptors field of the GFRα2-positive neurons in NRTN-OE mice. It is possible these neurons had an increased receptor field.

4.6 DICUSSION: FUNCTIONAL EFFECTS OF NRTN OVEREXPRESSION

4.6.1 Behavioral response in menthol water aversion test

Most studies on the role of growth factors in regulating survival and maintenance of nociceptors have been done in DRG neurons. However, for reasons that are not clear, the epithelial-driven overexpression of growth factors is often found to have more dramatic effects on trigeminal afferents than DRG afferents. For example, NRTN induced hypertrophy of GFRα2-positive neurons was more dramatic in TG than in DRG. In ARTN-OE mice, the percentage increase of mRNA level of GFRα3, TRPV1 and TRPA1 are nearly doubled in TG than in DRG (Elitt et al., 2006; Elitt et al., 2008). In addition, persistent pain involving cranial nerves is of major clinical significance and is understudied. Therefore, we examined trigeminal neurons in parallel with our studies of cutaneous DRG neurons. The tongue is one of the well-

characterized sensory organs that routinely contacts noxious thermal and chemical stimuli. For the anterior 2/3 of the tongue, general sensory information is conveyed via the lingual nerve (a branch of mandibular division of trigeminal ganglion) to the trigeminal nucleus, whereas taste information is conveyed via the chorda tympani (a branch of cranial nerve VII) to the nucleus of the tractus solitarious. GFRα2 is expressed in TG in numbers similar to that found in DRG (as shown in Fig. 18). In vitro, NRTN promotes the nerve regrowth of the geniculate ganglion neurons, and GFRα2 mRNA is expressed in these neurons (Yamout et al., 2005).

The drinking aversion test in the NRTN-OE mice showed that these mice were hypersensitive to menthol, the ligand for TRPM8. Our real-time RT PCR analysis showed an 84% increase in TRPM8 mRNA in TG. Several groups have characterized TRPM8 expression in TG and tongue. In rat, ISH showed TRPM8-expressing neurons were more abundant in TG than in DRG (35.3 ± 2.8% vs. 22.8 ± 6.9%). More than 40% of these TRPM8-positive neurons were in the mandibular division innervating the tongue (Kobayashi et al., 2005). Another group, using TRPM8 immunohistochemistry, showed that in rat TG, TRPM8 is expressed in 11.8 ± 1.3% of neurons (Abe et al., 2005). The difference in the percentage of TRPM8 expression in TG between these two groups might relate to the greater sensitivity of ISH relative to immunostaining. Abe et al. (2005) also showed that TRPM8 fibers are present in lingual nerve fibers innervating tongue. GFRα2 is also expressed in lingual ganglia (Nosrat, 1998) and in the fibers that innervate the taste buds of tongue (Kawakoshi et al., 2005). Therefore, NRTN increased TRPM8 expression in GFRα2 neurons in TG and there are more TRPM8 fibers in lingual nerve that project to tongue, thus increased the oral sensitivity to menthol. Since we found that GFRα2-positive TG neurons are hypertrophied in NRTN-OE mice, these neurons, like DRG neurons, may have hyperinnervation into their peripheral target, tongue. This could

also explain the increased oral sensitivity. Further characterization of TRPM8 expression in tongue in NRTN-OE mice will be helpful to explain the behavioral changes.

4.6.2 Changes in sensitivity to cool and cold stimuli

The two-temperature choice test showed that overexpression of NRTN increased the sensitivity to cool temperatures (17ºC and 20ºC). This may be due to the increase of TRPM8 expression in the DRG of NRTN-OE mice. In NRTN-OE mice most of the TRPM8-positive neurons also express GFRα2-immunoreactivity and these neurons are hypertrophied and appeared to hyperinnervate the skin. It is well established that TRPM8 can be activated by cooling temperature between 18-25ºC. Behavioral experiments in TRPM8 KO mice provided evidence that TRPM8 is required for avoidance of innocuous cold (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Our results here further confirm the ability of TRPM8 to detect cooling sensation and indicate that TRPM8 expression can be regulated by NRTN.

TRPA1 mRNA level is increased in NRTN-OE DRG and TG. One would expect NRTN-OE mice to have increased sensitivity to noxious cold. In physiology study, the CMCH fibers in NRTN-OE mice had significantly lower cold threshold (12.26 ± 1.19 °C) comparing with those in WT mice (16.17 ± 1.65 °C). These results indicated the NRTN overexpression increased sensitivity to noxious cold of CMCH fibers and this is correlated with an increase of TRPA1 expression in DRG. However, behavioral tests showed NRTN-OE mice had decreased sensitivity to -20ºC ice plate. One explanation is that TRPA1 is not activated at -20ºC and what channel or receptor that underlies this phenotype is not clear. Previous electrophysiological recording made from fibers innervating hairy skin of rat hindpaw had shown that almost all fibers were activated when temperature is below 0°C (Simone and Kajander, 1996). Since NRTN-OE mice had

increased fiber innervation density into the skin, one would expect that NRTN-OE mice had increased sensitivity to -20°C ice plates. However, central circuits are involved in a behavioral response, so the decreased behavioral sensitivity to -20°C ice plates may be the result of compensation via central circuits, for example in the spinal cord, that prevents the increase in afferent sensitivity from being expressed behaviorally. Previous studies of NGF-OE and GDNF-OE mice found changes in gene expression in the spinal cord that have the potential of decreasing efficacy of afferent input (Molliver et al., 2005).

4.6.3 NRTN overexpression increases calcium signaling in response to menthol

Calcium imaging studies of cutaneous afferents support both the anatomical and behavioral results with respect to the increase in menthol/cool sensitivity. The apparent increase in the number of menthol responsive neurons seen with calcium imaging further supports the hypothesis that NRTN increases the number of TRPM8-positive afferents innervating the skin. However, NRTN does not increase the magnitude of the response to menthol even though multiple concentrations were tested. As discussed previously in section 4.5.3, there are two distinct population of neurons that express TRPM8 in NRTN-OE DRG. One is the small diameter, IB_4-negative neurons, and this population also expresses TRPM8 in WT DRG. The second population is GFRα2-positive, IB_4-binding neurons, the expression of TRPM8 in these neurons can only be detected in NRTN-OE but not WT DRG. IB_4-488 injection through the hind paw only labeled the second population of neurons. Thus the increase in the number of menthol-

response neurons in our study is attributed to an increase of TRPM8 expression in GFRα2-positive neurons in NRTN-OE DRG.

4.6.4 Effect of NRTN on detection of noxious heat stimuli

Previous studies using isolated DRG neurons from GFRα2 KO mice showed that NRTN/GFRα2 signaling contributed to detection of noxious heat. Heat-evoked inward current was significantly reduced in IB_4-binding neurons in GFRα2 KO mice (Stucky et al., 2002). Since GFRα2 expression level is increased in the DRG of NRTN-OE mice, one would expect an increased heat sensitivity of NRTN-OE mice. However, our behavioral studies show that overexpression of NRTN does not alter the behavioral response to noxious heat in Hargreaves test. We also performed intracellular recordings using the *ex vivo* preparation, and hypothesized that overexpression of NRTN would increase the C-fiber sensitivity to noxious heat. However, heat sensitivity and thresholds were not changed in NRTN-OE mice. There are two possible explanations for the results. First, although *in vitro* physiological study showed a decrease of heat sensitivity of DRG neurons in GFRα2 KO mice (Zwick et al., 2002), in vivo behavioral tests indicated that these mice had normal response to innocuous warm and noxious heat in hot plate test. Thus the role of NRTN/GFRα2 signaling in heat transduction is still unclear. Second, most GFRα2-positive neurons do not express the heat sensitive channel TRPV1 in WT mice (**Table 5**). The expression the TRPV1 are not changed in NRTN-OE mice (**Table 9**), suggesting that receptors other than TRPV1 transduce noxious heat in GFRα2-positive neurons. How NRTN ovexpression regulates the expression of heat sensitive receptors is still unclear.

4.6.5 Effects of NRTN on detection of mechanical stimuli

Previous studies in GDNF-OE mice showed that IB_4-binding C-fibers had increased mechanosensitivity that correlated with enhanced ASIC2 mRNA levels and increased immunostaining intensity in DRG neurons (Albers et al., 2006b). Overexpression of NRTN also increased ASIC2a mRNA level in DRG and therefore we performed von Frey test to determine if NRTN increased behavioral sensitivity to mechanical stimuli. NRTN-OE mice exhibited increased withdraw frequency compared with WT mice. Interestingly, we only observed an increase of ASIC2a, but not ASIC2b, that was increased in GDNF-OE mice (Albers et al., 2006b). Previous work by Garcia-Anoveros et al. (2001) demonstrated that ASIC2a can form homometric channels and does not require ASIC2b. ASIC2a is localized to low-threshold mechanosensitive DRG neurons and is transported to cutaneous mechanosensory terminals (Garcia-Anoveros et al., 2001). Thus, an increase in ASIC channel expression driven by elevated NRTN level may explain the enhanced mechanosensitivity of nociceptive afferents. The *ex vivo* preparation showed a significant increase in the number of CM fibers in NRTN-OE mice DRG. However, most of the CM fibers when tested *ex vivo* have a high mechanical threshold (>100mN) that is unlikely to be reached with the stimulation used in the behavioral test. As we discussed before, the additional CM fibers are recruited from the silent C-fibers that are activated by enhanced NRTN. These fibers can only detect intense mechanical stimuli and may not contribute to the behavioral response at all. The increased behavioral sensitivity could be due the increased size of the terminal field, increased peripheral innervation density and increased CV of DRG neurons. These factors will make for more effective temporal summation to the secondary neurons in dorsal horn.

4.7 CONCLUSION

In conclusion, we have shown that overexpression of NRTN in the epidermis induced hypertrophy of GFRα2-positive neurons in DRG and hyperinnervation of the skin by nonpeptidergic afferent fibers. This phenotype, together with the findings from GFRα2 KO mice (Lindfors et al., 2006), supports the hypothesis that NRTN is required for growth, differentiation and terminal branching of nonpeptidergic cutaneous nociceptors. TRPM8 expression is dramatically increased in GFRα2-positive neurons in the DRG and TG of NRTN-OE mice, which was correlated with behavioral sensitivity to menthol and cooling temperature. NRTN also increased ASIC2a expression in DRG, which may have contributed to appearance of additional CM fibers and/or the increase in behavioral sensitivity to mechanical stimuli.

Gene	Forward Primer(5'--3')	Reverse Primer(5'--3')
NRTN	TGAGGACGAGGTGTCCTTCCT	AGCTCTTGCAGCGTGTGGTA
GDNF	AGCTGCCAGCCCAGAGAATT	GCACCCCCGATTTTTGC
ARTN	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
NGF	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
GFRα1	GTGTGCAGATGCTGTGGACTAG	TTCAGTGCTTCACACGCACTTG
GFRα2	TGACGGAGGGTGAGGAGTTCT	GAGAGGCGGGAGGTCACAG
GFRα3	CTTGGTGACTACGAGTTGGATGTC	AGATTCATTTTCCAGGGTTTGC
ASIC2a	ATGGACCTCAAGGAGAGCCCCAG	AAGTCTTGATGCCCACACTCCTGC
ASIC2b	CGCACAACTTCTCCTCAGTGTTTAC	TTGGATGAAAGGTGGCTCAGAC
CGRP	TCAGCATCTTGCTCCTGTACCA	CTGGGCTGCTTTCCAAGATT
TRPV1	TTCCTGCAGAAGAGCAAGAAGC	CCCATTGTGCAGATTGAGCAT
TRPV3	TGAAAGAAGGCATTGCCATTT	GAAACCAGGCATCTGACAGGAT
TRPA1	GCAGGTGGAACTTCATACCAACT	CACTTTGCGTAAGTACCAGAGTGG
TRPM8	CGTGGGAGGGTGTCATGAAG	GTTGTCGTTGGCTTTCGTGTT
p75	GGGTGATGGCAACCTCTACAGT	GTGTCACCATTGAGCAGCTTCT
TrkA	AGAGTGGCCTCCGCTTTGT	CGCATTGGAGGACAGATTCA
$P2X_3$	TGGAGAATGGCAGCGAGTA	ACCAGCACATCAAAGCGGA
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT

Table 6. Primer sequences used for real-time RT PCR assays.

	WT	NRTN-OE
Number of myelinated axons	556.25 ±14.64	550.5 ± 21.17
Number of unmyelinated axons	2472.25 ± 83.78	2430.25 ± 44.95
Diameter of myelinated axons(μm)	3.64 ± 0.11	3.92 ± 0.23*
Diameter of unmyelinated axons(μm)	0.86 ± 0.04	1.12 ± 0.05*
Myelin thickness (μm)	0.65 ± 0.03	0.70 ± 0.03

Table 7. Number and diameter of myelinated and unmyelinated fibers in the saphenous nerve of WT and NRTN-OE mice.

T-test, n=4, *p<0.05

Gene Assayed	Percentage Change		
	Flank skin	Glabrous skin of hindpaw	Hairy skin of hindpaw
NRTN	+313% *	+1123%*	+485%*
GDNF	-73% *	-56%*	-67%*
Artemin	+14%	+8%	+12%

Table 8. Change in mRNA level of growth factors in skin of NRTN-OE mice compare with WT mice.

T-test, n=4,*p<0.05

Gene Assayed	Percentage Change (in DRG)	Percentage Change (in TG)
GFRα1	+6%	-17%
GFRα2	+62% *	+98% *
GFRα3	-32%*	-28%*
Ret	+77%*	+144%*
P2X3	+84% *	+42%*
TRPM8	+44%*	+84%*
TRPA1	+74%*	+42%*
ASIC2a	+95%*	+125%*
GluR5	-80%*	-58%*
CGRP	25%	-29%
TRPV1	-22%	-10%
TRPV3	0%	-13%
P75	-5%	-11%
ASIC2b	20%	13%
TrkA	-23%	-13%

Table 9. Change in mRNA level of receptor and TRP channel genes in lumbar DRG and TG of NRTN-OE mice compare with WT mice.

T-test, n=4,*p<0.05

	Men+/IB4+	Cap+/IB4+
WT (n=3)(%)	14.02±1.38	30.22±0.31
NRTN-OE (n=3)(%)	48.81±1.68*	32.29±1.47

Table 10. NRTN enhances the percentage of IB_4-488 labeled cutaneous sensory neurons that respond to menthol.

We performed calcium imaging analysis to examine the percentage of IB_4-488 labeled neurons that respond to menthol or capsaicin. The percentage of IB_4-488 labeled neurons that respond to menthol significantly increased from 14.02 ± 1.38 to 48.81 ± 1.68. Whereas those respond to capsaicin did not change. Fisher's exact test, *p<0.01.

Fiber type		CV(m/s)	
WT	**NRTN-OE**	**WT**	**NRTN-OE**
AM(n=10)	AM(n=14)	13.48±2.02	13.92±1.50
CC(n=1)	CC(n=4)	0.59	0.87±0.12
CH(n=14)	CH(n=13)	0.33±0.01	0.47±0.02*
CM(n=10)	CM(n=20)	0.55±0.02	0.68±0.02*
CMH(n=41)	CMH(n=37)	0.57±0.01	0.67±0.02*
CMCH(n=23)	CMCH(n=20)	0.52±0.01	0.62±0.02*

Table 11. The conduction velocity of C-fibers in saphenous nerve is significantly increased in NRTN-OE mice.

T-test, *p<0.05. Collaboration with Dr. Richard Koerber's lab

Threshold(mN)	WT-CMH/C	NRTN-OE-CMH/C	WT-CM	NRTN-OE-CM
1--5	21	11	3	1
10--25	30	23	3	1
50--100	11	8	0	2
>100	2	2	4	16
Total	64	57	10	20

Table 12. Mechanical threshold of C-fibers in WT and NRTN-OE mice.

This table shows the number of C-fibers that has mechanical threshold that fall in each category. Collaboration with Dr. Richard Koerber's lab

	CH	CMH	CMHC
WT(°C)	41.77±1.56 (n=14)	42.32±0.72(n=41)	41.93±1.16(n=23)
NRTN-OE (°C)	42.67±1.20 (n=13)	43.26±0.73(n=37)	42.07±1.21(n=20)

Table 13. Heat threshold of C-fibers.

The heat threshold of CH, CMH and CMHC are not changed in NRTN-OE mice. Two-way ANOVA, df = (1, 2), $F = 6.43$, $p = 0.13$. Collaboration with Dr. Richard Koerber's lab

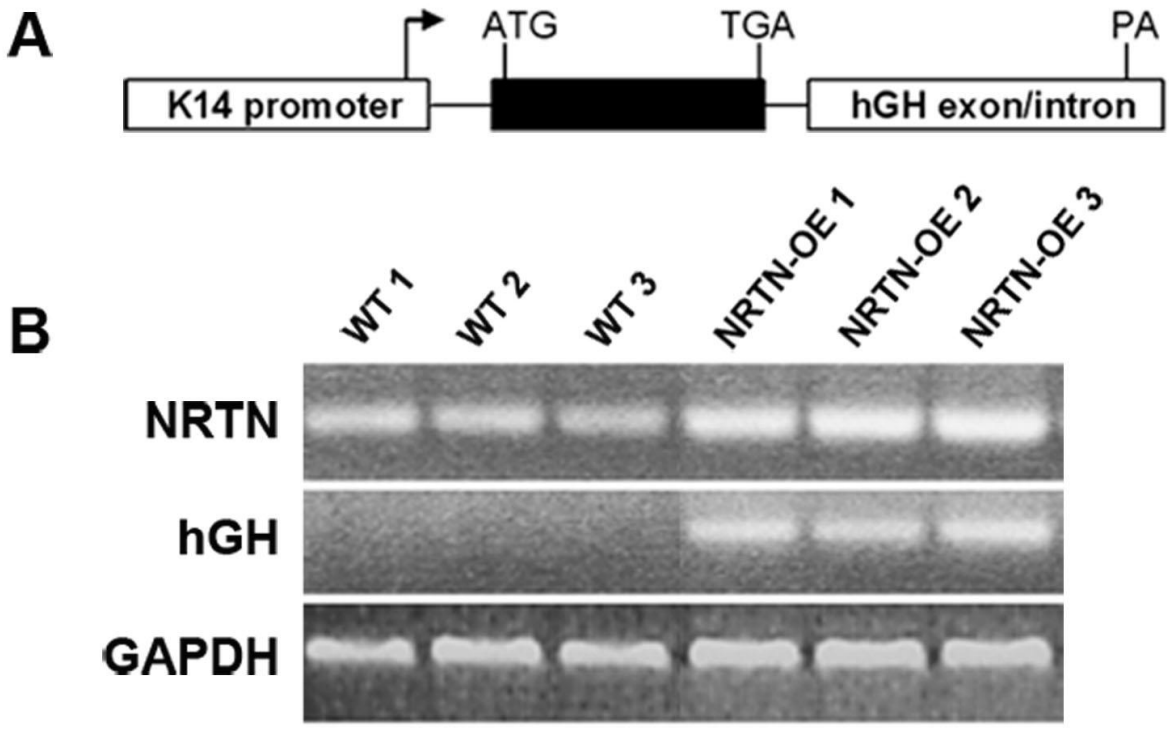


Figure 16. Overexpression of NRTN in the skin is driven by the K14 keratin promoter.

(***A***), Diagram of transgene construct used for isolation of NRTN-OE mice. The K14 promoter drives expression of NRTN sequence represented by black boxes. The 3' human growth hormone sequence provides splice sites and a poly (A) addition signal. The arrow indicates the transcription start site, and ATG and TGA are translation start and stop sites, respectively. (***B)***, RT-PCR analysis of RNA isolated from WT (*n*=3) and transgenic (NRTN-OE; *n*=3) back skin showing increased expression of NRTN mRNA in NRTN-OE skin. Note significant enhancement in the level of NRTN mRNA in NRTN-OE skin and lack of transgene expression (hGH, Human growth hormone) in WT skin samples. Collaboration with Dr. Kathryn Albers lab.

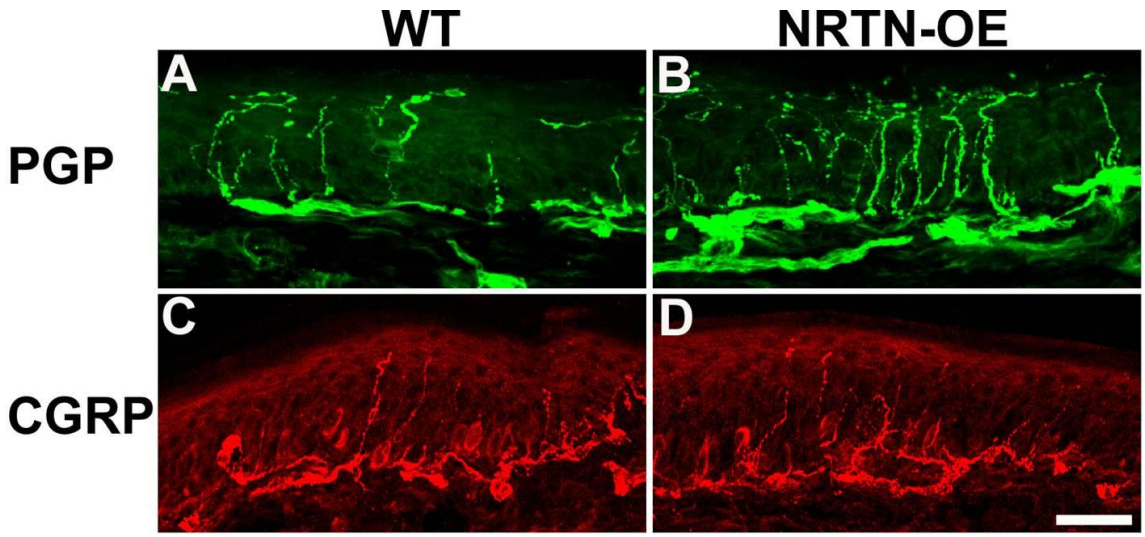


Figure 17. Overexpression of NRTN induced hyperinnervation of PGP9.5-positive but not CGRP-positive free nerve endings in the epidermis of footpad glabrous skin.

Immunolabeling of glabrous skin from WT (left) and NRTN-OE (right) mice using an antibody against PGP9.5 (***A, B***) and CGRP (***C, D***). Scale bar=25µm.

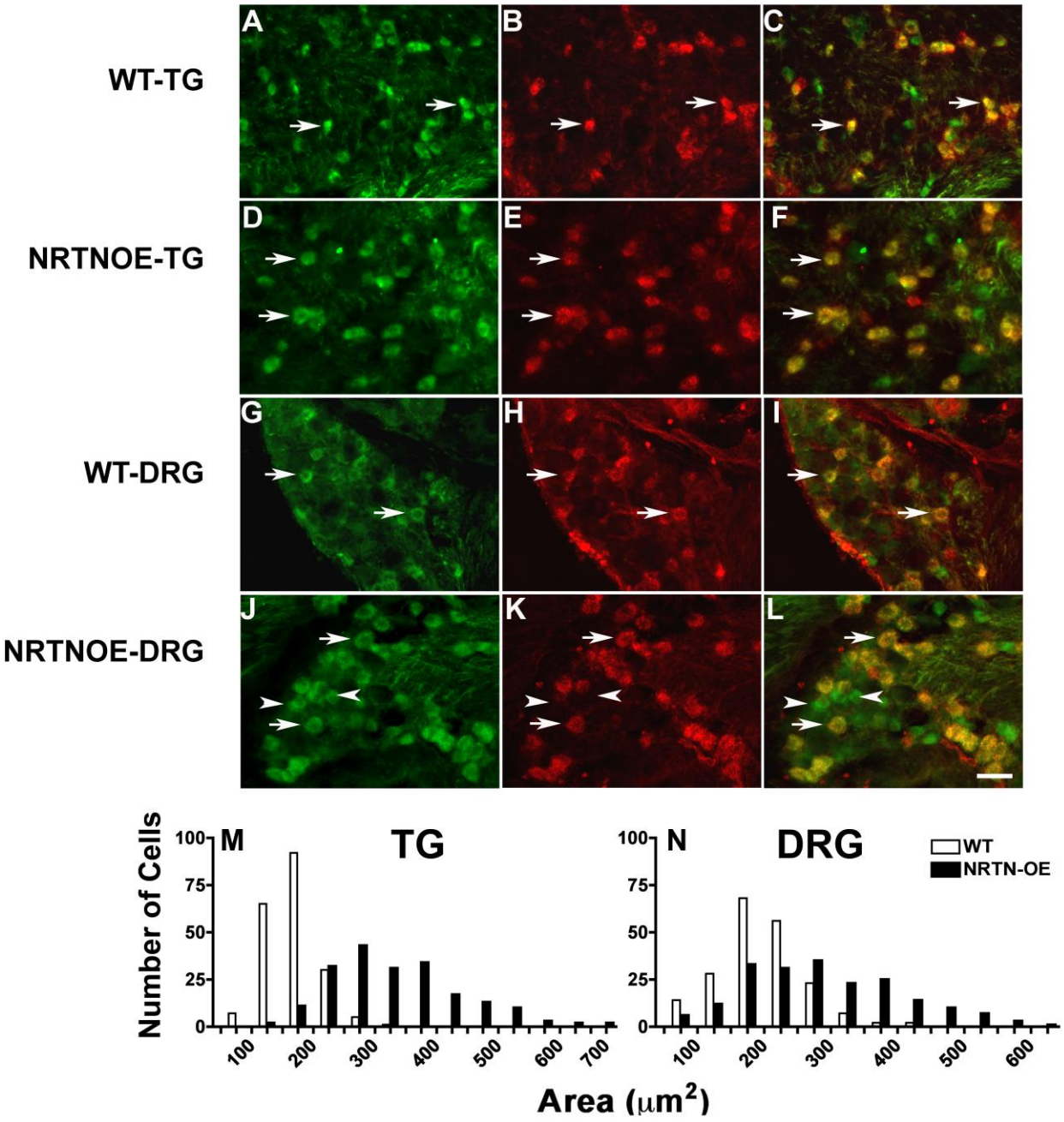

WT-TG
NRTNOE-TG
WT-DRG
NRTNOE-DRG
A
B
C
D
E
F
G
H
I
J
K
L
M
TG
N
DRG
WT
NRTN-OE
Number of Cells
100
75
50
25
0
100
200
300
400
500
600
700
Area (μm²)

Figure 18. Sensory neurons responsive to NRTN are hypertrophied and bind IB_4.

GFRα2 immunolabeling (green) of WT (***A, C***) and NRTN-OE (***D, F***) TG show GFRα2-positive neurons are larger in size. Nearly all GFRα2-positive neurons in WT (***B***) and NRTN-OE (***E***) bind IB_4 (arrow). The size distribution of GFRα2-positive neurons in TG are shown in ***M***. There is a rightward shift in the distribution, indicating that GFRα2-positive neurons in TG are hypertrophied. DRG neurons in NRTN-OE mice show a similar hypertrophy of GFRα2-positive neurons (***J, L***) compared with WT DRG (***G, I***). Plots of the size distribution of GFRα2-positive neurons in DRG (***N***) show a significant rightward shift. Chia squared test, for DRG, Chi squared=156.973, df = 11, $p< 0.0001$. For TG, Chi squared=2717.693, df = 12, $p< 0.0001$.

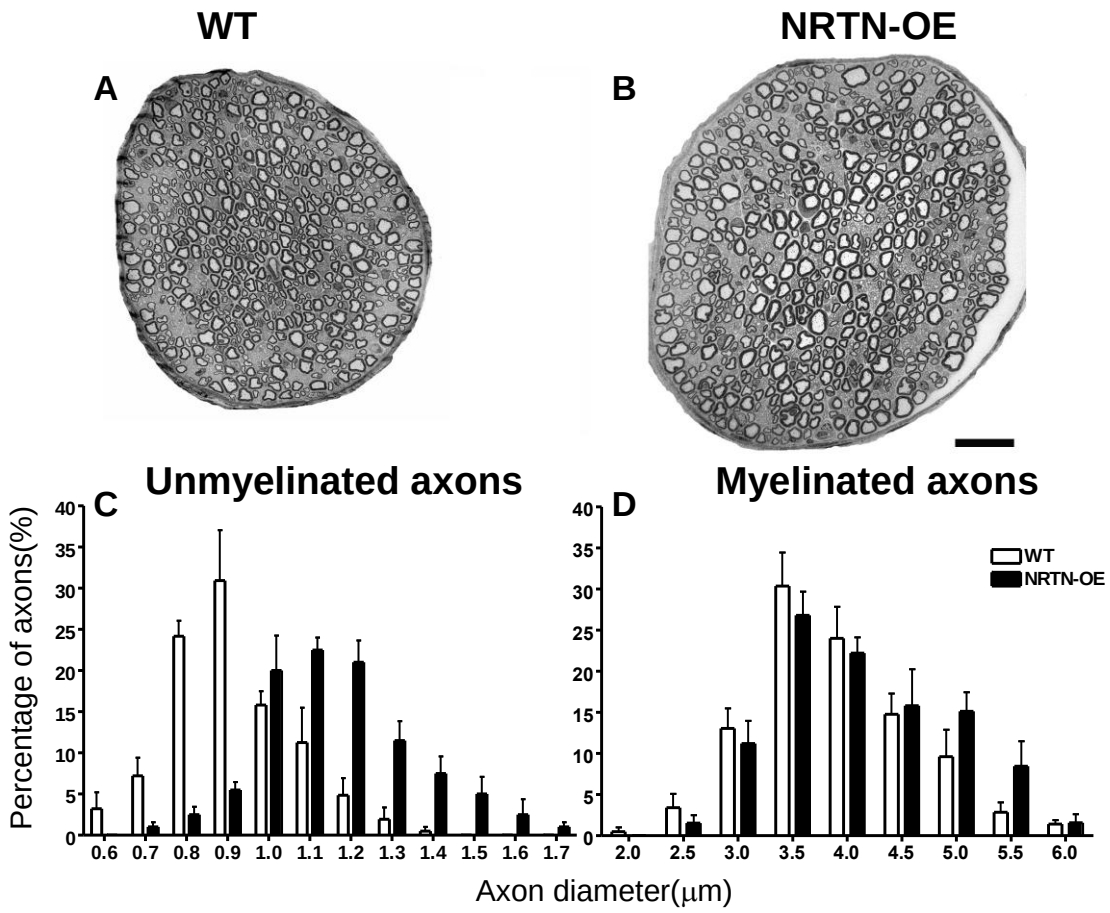


Figure 19. NRTN-OE mice have larger-diameter unmyelinated axons in cutaneous nerves.

A and ***B***, low magnification electron microscopic montages of saphenous nerve cross-sections at mid-thigh level from a WT (***A***) and NRTN-OE (***B***) mouce. Scale bar=20μm. Note that although the total number of axons is not changed (see **Table 7**), the nerves of NRTN-OE are larger than those from WT. ***C*** and ***D***, histograms show the distribution of axon diameters of unmyelinated (***C***) and myelinated (***D***) axons in saphenous nerves from WT (*n*=4) and NRTN-OE (*n*=4) mice. The distribution of unmyelinated axons has a significant rightward shift. One-way ANOVA.

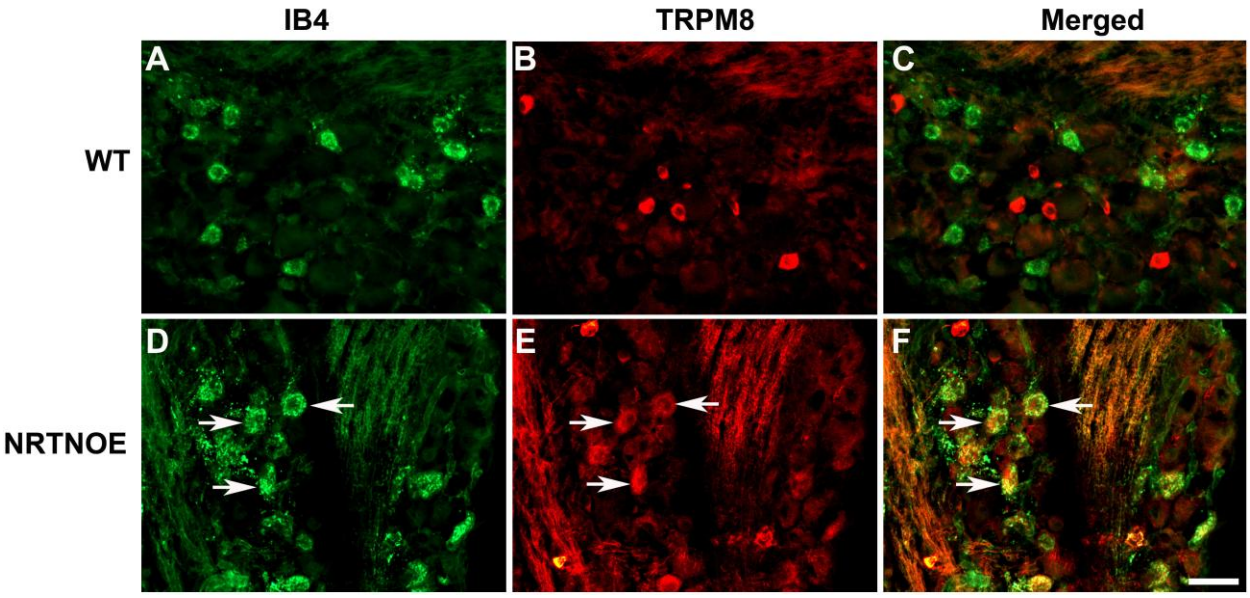


Figure 20. In NRTN-OE mouse DRG, more IB_4-binding neurons express TRPM8.

In WT DRG (***A-C***), IB_4-binding neurons do not express TRPM8, whereas in NRTN-OE DRG (***D-F***), most IB_4-binding neurons express TRPM8 (arrows). Scale bar=20µm.

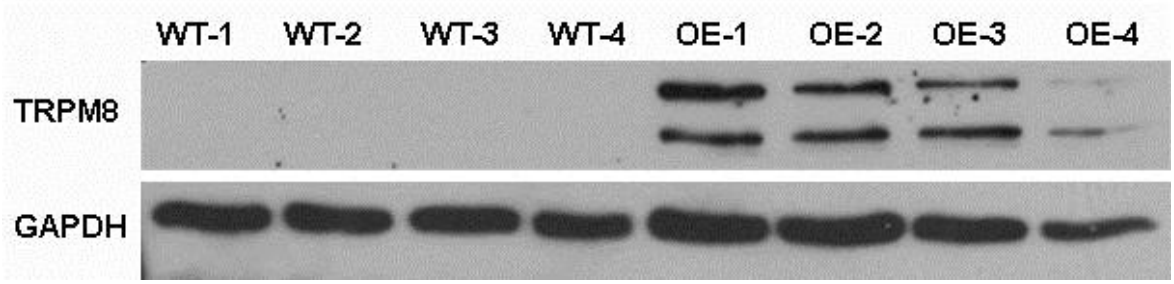


Figure 21. Western blotting shows increased TRPM8 protein in NRTN-OE DRG.

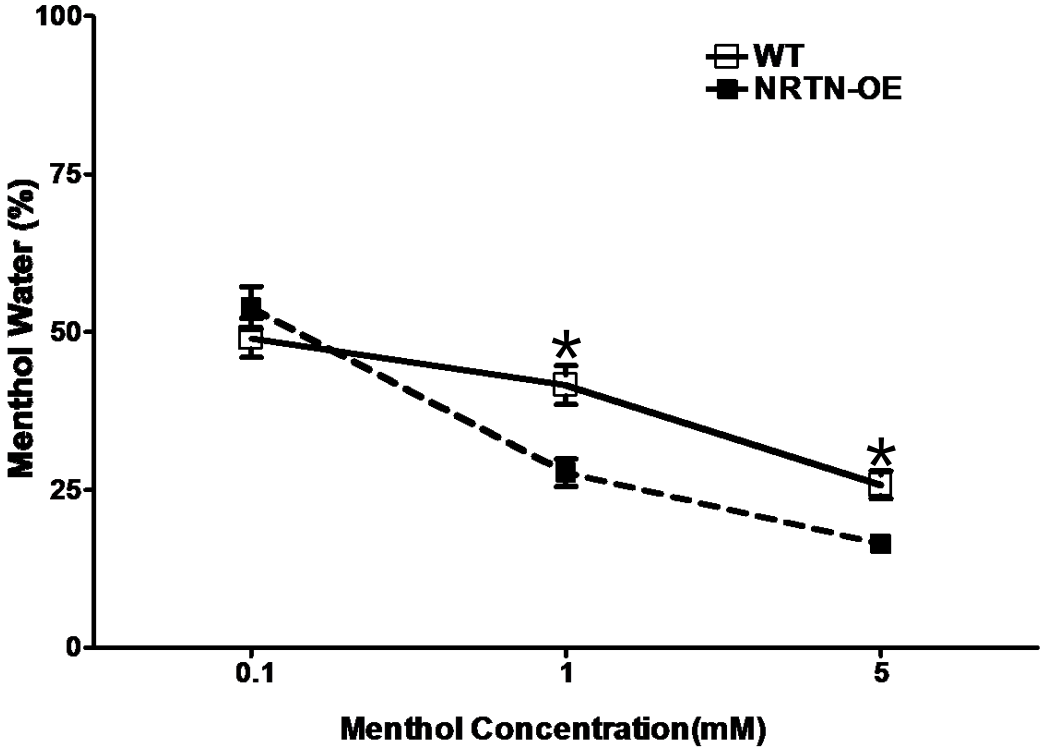


Figure 22. NRTN-OE mice display oral sensitivity to menthol.

Six WT and 6 NRTN-OE mice were tested for oral sensitivity to menthol using a two-bottle drinking aversion assay. One bottle contained water with vehicle (0.07% ethanol) and the other bottle had menthol at three different concentrations. At low concentration (0.1 mM), both WT and NRTN-OE mice drink equal amount of water from each bottle and show no preference. As the menthol concentration increased, both WT and NRTN-OE mice drank less menthol water, with NRTN-OE mice drinking less menthol water than WT mice. Two way ANOVA, df = (1, 2), F=31.76, *p<0.05, Bonferroni Post-hoc test, n=6 for each genotype.

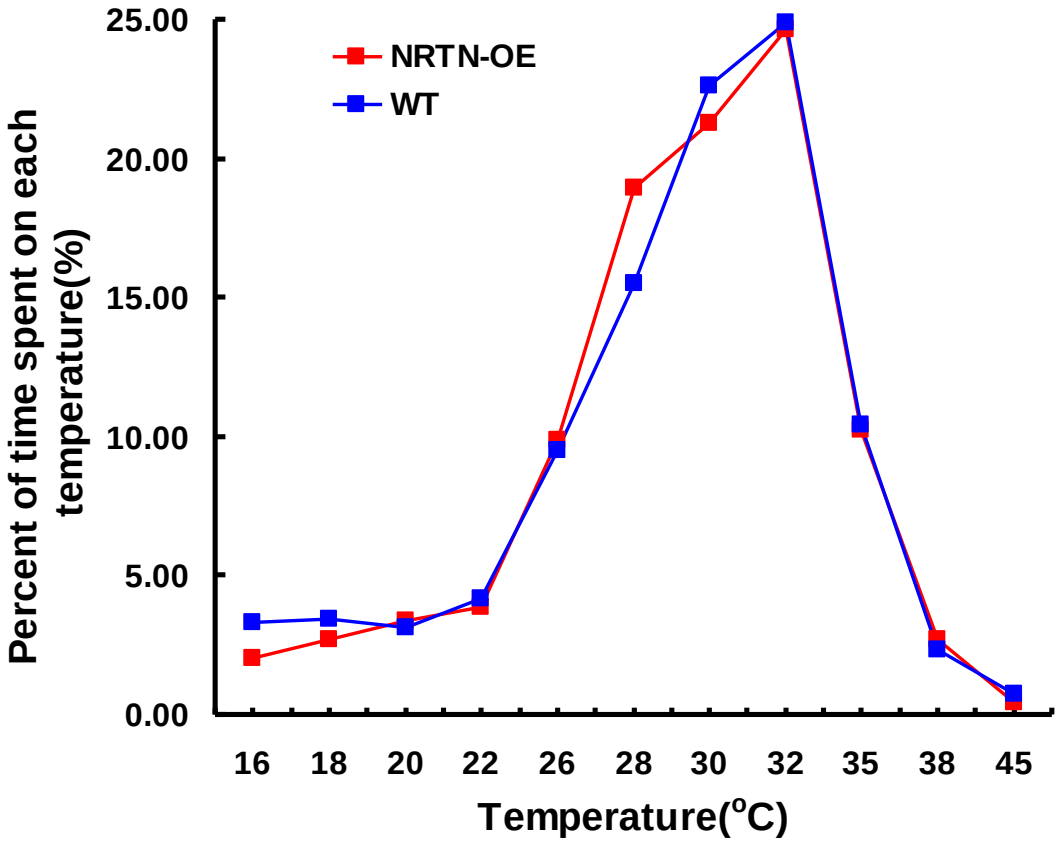


Figure 23. Both WT and NRTN-OE mice spent most of their time on 32°C key in a thermal gradient test.

In the thermal gradient test, mice were allowed to explore 11 keys with temperature ranging from 16°C to 45°C for 60 mins. The time mice spent on each key in the 30-60 mins were recorded and percent of time spent on each key were calculated and show in figure. Two way ANOVA, df = (1, 10), *F*= 4.14E-11, *p=0.99*, *n=20* for each genotype.

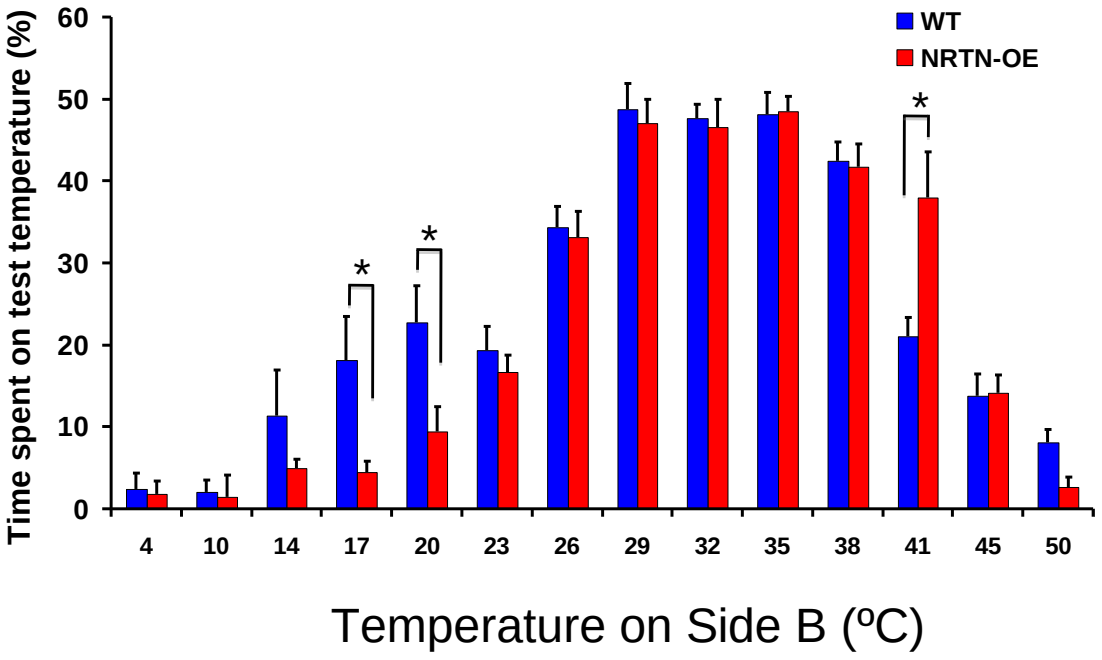


Figure 24. NRTN-OE mice have increased sensitivity to 17ºC and 20ºC, decreased sensitivity to 41ºC.

Mice were allowed to explore two adjacent keys for 5 mins, the temperature of side A was held at 32ºC and side B was ranged from 4 to 50ºC (For method, see **Fig. 7**). The percentage of time spent on side B was calculated. When temperature on side B is 32ºC, mice spent equal time on both sides, showed 50% time on side B. When the temperature of side B decreased, mice spent lass time on side B, however when temperature of side B was 17ºC or 20ºC, NRTN-OE mice spent significantly less time on side B comparing with WT, indicating NRTN-OE mice had increased sensitivity to 17ºC and 20ºC. NRTN-OE mice also spent significantly more time on 41ºC plate, indicating NRTN-OE mice had decreased sensitivity to 41ºC. Two way ANOVA, *$*p<0.05$;$n=20$* for each genotype.

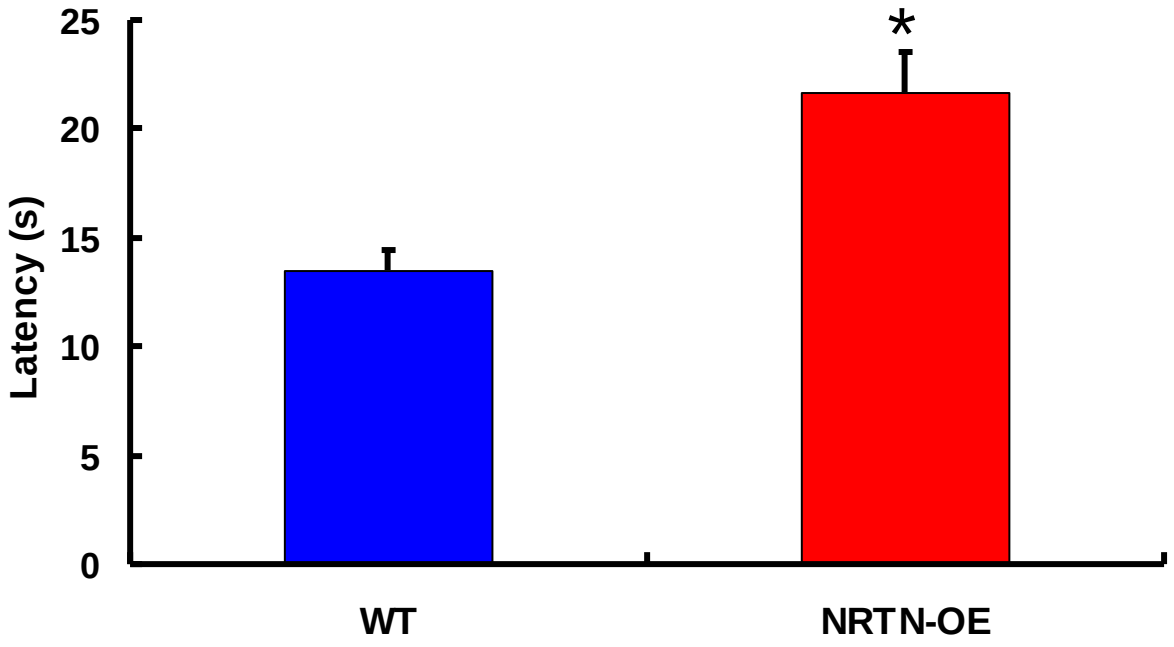


Figure 25. NRTN-OE mice have significantly longer response latency on -20ºC ice block test.

Mice were placed in plexiglass container with an ice floor and the temperature was maintained at -20ºC (the ice block is placed on top of a dry ice block so that the temperature can be maintained at -20ºC). The latency to the first nocifensive responses such as foot lifting or jumping was shown in figure. NRTN-OE mice had significantly longer latency compared with WT mice. T-test, *$*p<0.05$;$n=20$* for each genotype.

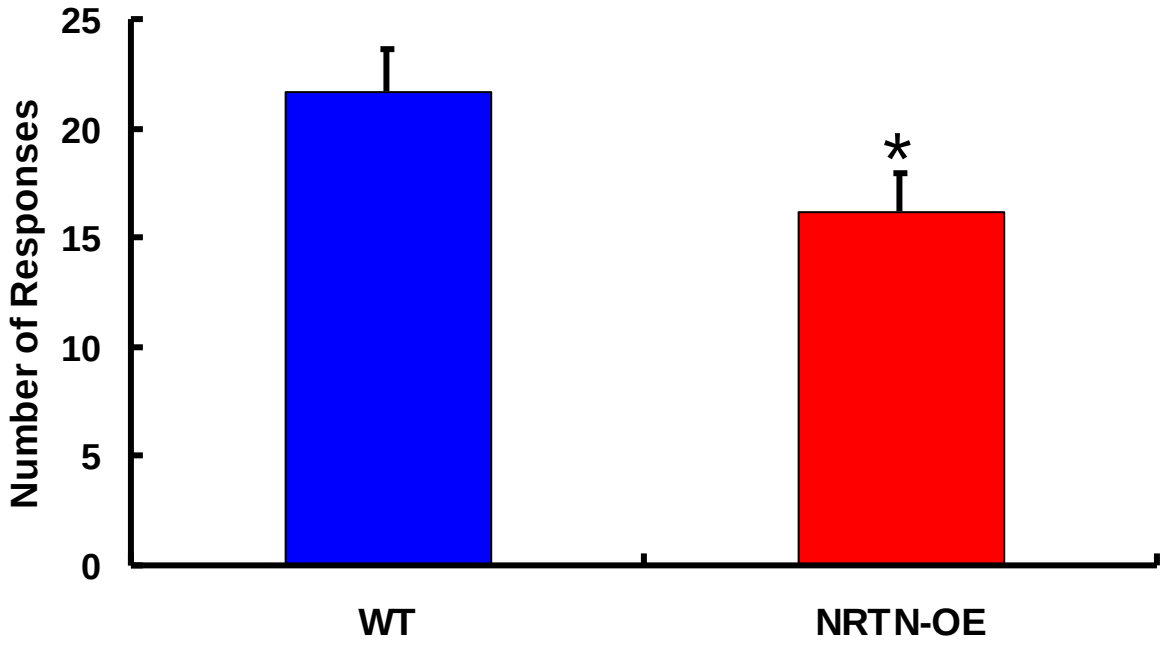


Figure 26. NRTN-OE mice have significantly less number of behaviors in 60s on -20ºC ice block test.

Mice were placed in plexiglass container with an ice floor and the temperature was maintained at -20ºC (the ice block is placed on top of a dry ice block so that the temperature can be maintained at -20ºC). The number of nocifensive responses such as foot lifting or jumping in the first 60s was counted and shown in figure. NRTN-OE mice had significantly less number of behaviors compared with WT mice. T-test, *$*p<0.05$;$n=20$* for each genotype.

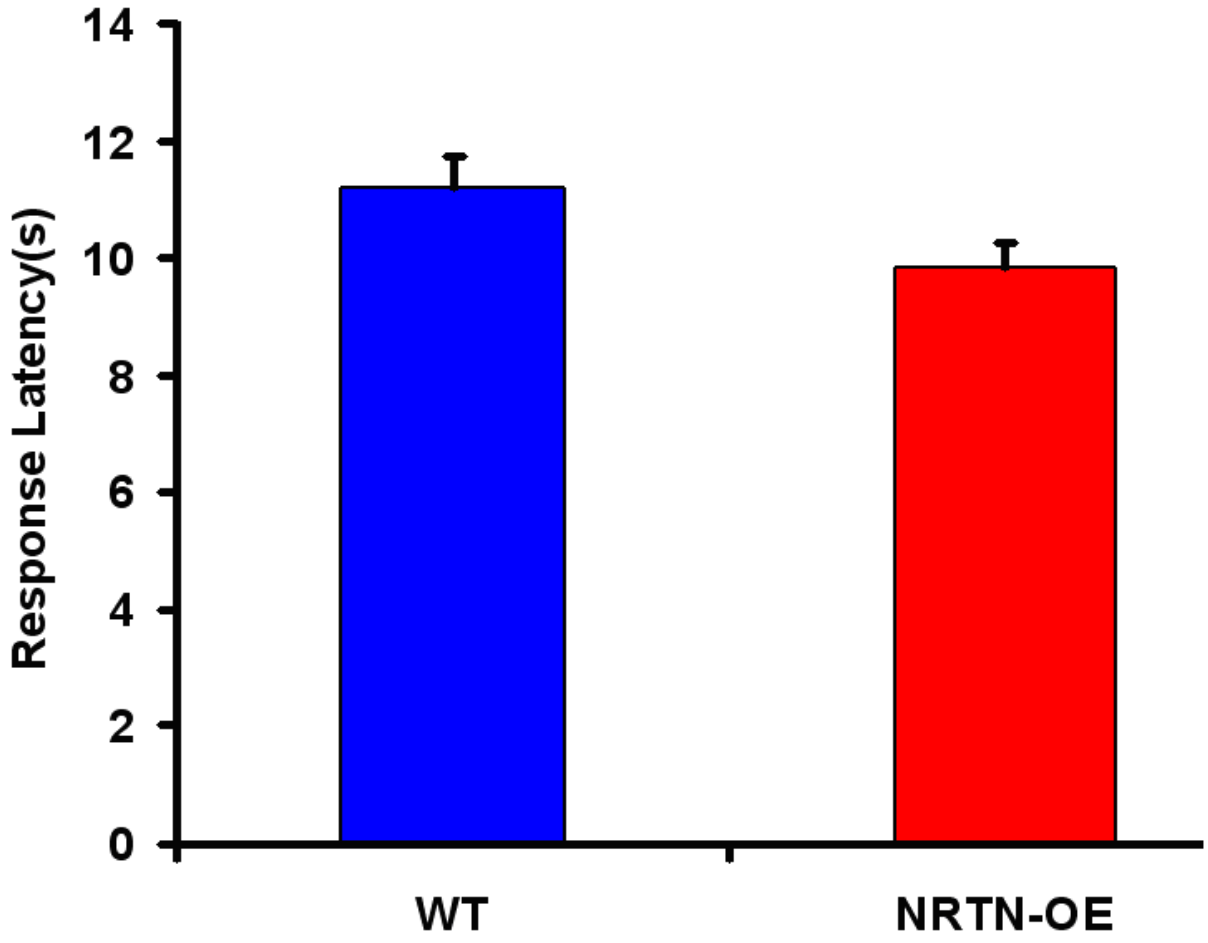


Figure 27. NRTN overexpression does not alter the behavioral sensitivity to noxious heat.

There is not significantly difference of the withdrawl latency between WT and NRTN-OE mice in Hargreaves test. T-test, *p=0.055; n=20* for each genotype.

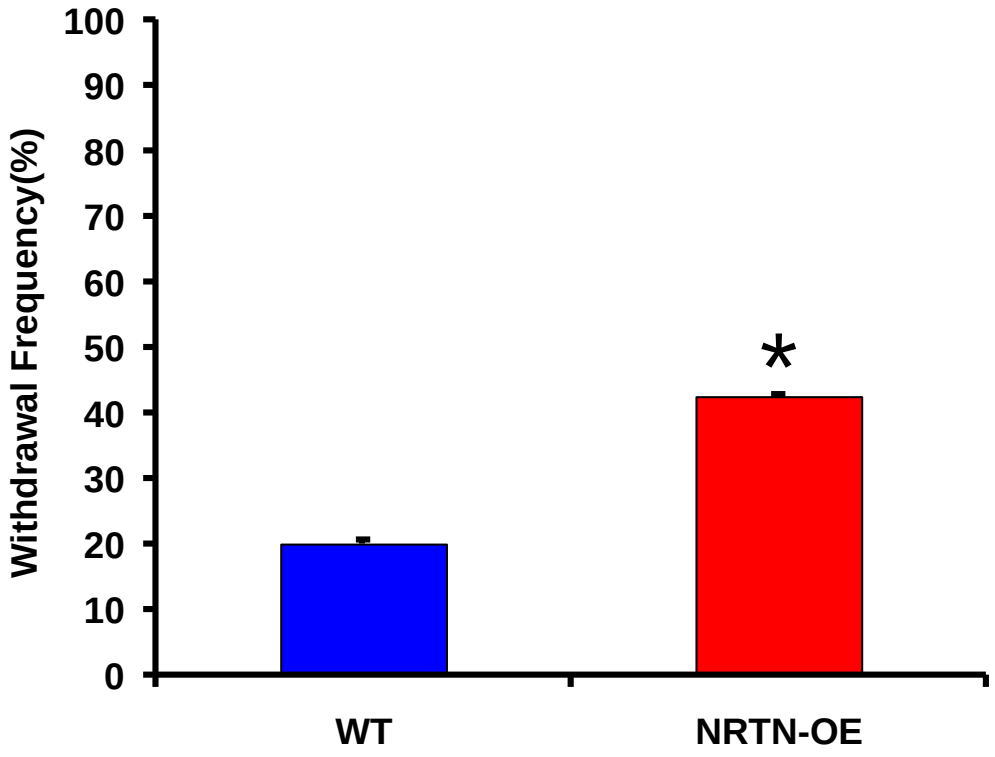


Figure 28. Overexpression of NRTN in skin increases mechanical sensitivity.

Mechanical sensitivity was assessed using von Frey filaments. Before CFA injection, NRTN-OE mice had increased withdraw frequency compared with WT. T-test, *$p<0.05$, $n=8$ for each genotype.

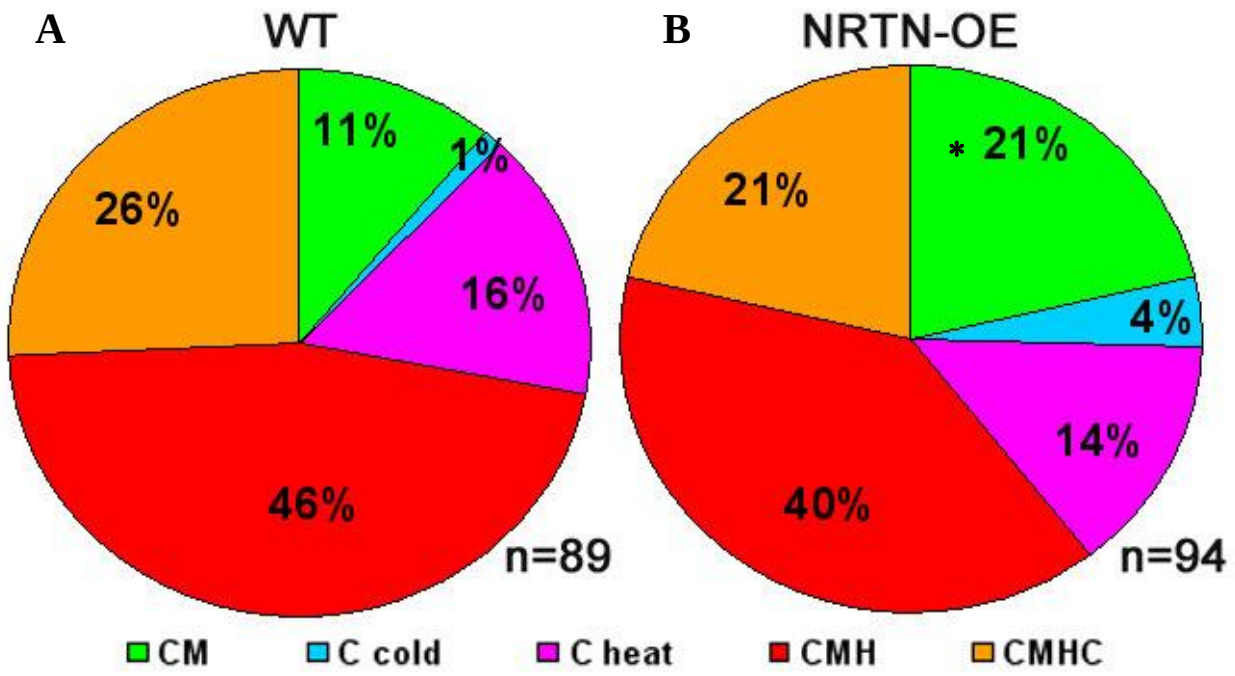


Figure 29. The percentage of CM neurons was increased in NRTN-OE mice in saphenous nerve.

A) Distribution of C fibers in WT mice DRG. (*B*) In NRTN-OE mice, the percentage of CM fibers significantly increased, however the percentage of other C-fibers were not changed. Fisher's exact analysis, $*p<0.01$. Collaboration with Dr. Richard Koerber's lab

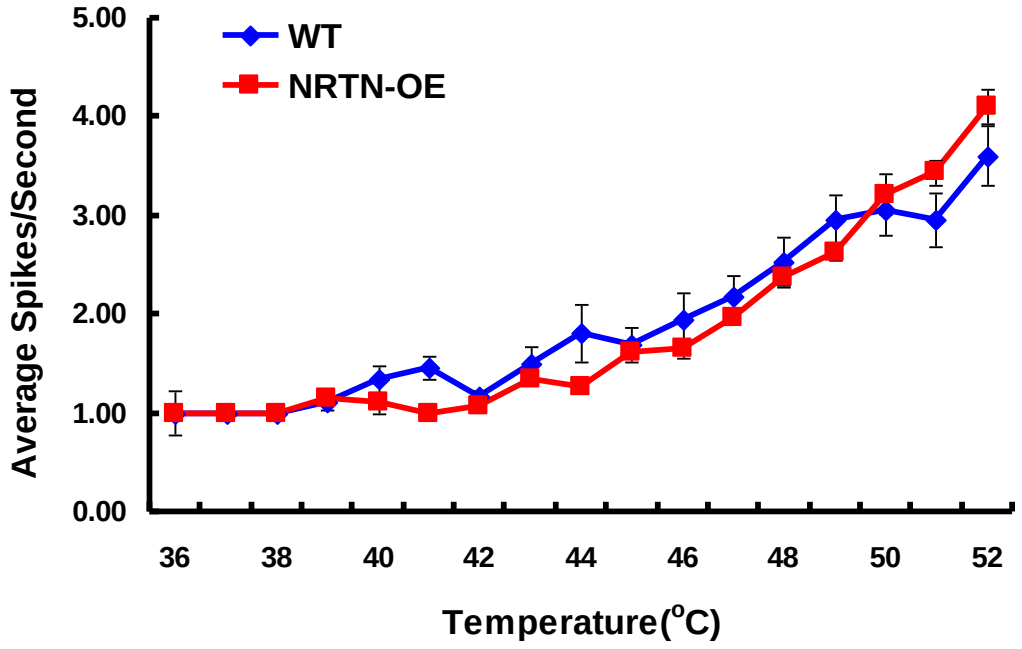


Figure 30. NRTN did not change heat sensitivity of CMH and CMCH fibers.

The firing frequency (average spikes per second) over a range of temperatures was calculated. Two way ANOVA, df=(1,16), F=0.02, p=0.89. Collaboration with Dr. Richard Koerber's lab

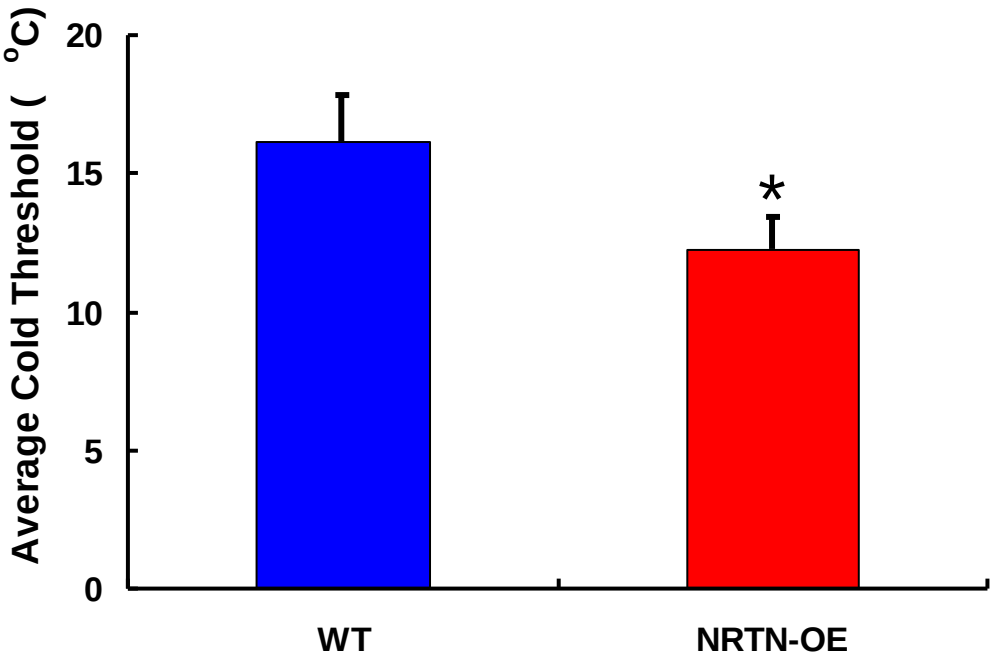


Figure 31. The cold threshold of CMCH fibers is decreased in NRTN-OE mice.

The average cold threshold of CMCH fibers decreased from 16.17 ± 1.65 °C in WT (n=23) to 12.26 ± 1.19 °C in NRTN-OE mice (n=20). T-test, *p<0.05. Collaboration with Dr. Richard Koerber's lab

5.0 GENERAL DISCUSSION

5.1 EVOLUTION OF GDNF FAMILY LIGANDS AND RECEPTORS

Four different GDNF family ligand-receptor (GFRα) pairs exist in mammals: GDNF-GFRα1, NRTN-GFRα2, ARTN-GFRα3 and PSPN-GFRα4. Phylogenetic analysis indicates that orthologs of all four GFRα receptors are present in all vertebrate classes from bony fish to human (Hatinen et al., 2007). However, not all vertebrate classes have all four orthologs of the GFLs. Clawed frog (*Xenpus tropicalis*) and chicken genomes contain only three GFLs (Hatinen et al., 2007). An ortholog of NRTN is missing from the frog genome, although frogs have a GFRα2 receptor. Evolutionary tracing and homolog modeling indicate that GDNF acts as the endogenous ligand of GFRα2 in frog. This finding supports the suggestion that there is overlap in the function of NRTN and GDNF and that there may be cross talk between GDNF with GFRα2 in mammals (Baloh et al., 2000a; Baloh et al., 2000b). The chicken genome lacks an ortholog of PSPN, but has a GFRα4 receptor that is able to signal via human PSPN (Enokido et al., 1998; Hatinen et al., 2007) suggesting that signaling via GFRα4 is important. The bony fish genomes have more than four homologous genes encoding GFLs. The Zebrafish genome contains two GDNF, two NRTN, one ARTN and one PSPN ortholog (Hatinen et al., 2007). The existence of multiple copies is thought to be the result of evolutionary chromosomal duplication

events. In fact, it is via chromosomal duplication events, which occur occasionally genome-wide, that the GFL family came to contain four different, but related members (Hatinen et al., 2007).

All GDNF family ligands originate from an ancient ProtoGFL gene (Chang et al., 2002). The ProtoGFL is thought to have given rise first to GDNF-like and NRTN-like genes. It has been proposed that the NRTN-like gene duplicated and that the two copies evolved into NRTN and PSPN/ARTN like genes. Duplication of the PSPN/ARTN like gene is then thought to have allowed the separate evolution of PSPN and ARTN, establishing the modern four gene, GFL family. Thus the GDNF and NRTN subgroup emerged before ARTN and PSPN subgroup (Hatinen et al., 2007). The fact that the sequence similarity between GDNF and NRTN is higher than that between NRTN and ARTN might explain why some neurons appear to express both GFRα1 and GFRα2, but coexpression of GFRα2 and GFRα3 in peripheral sensory neurons is rare (Horger et al., 1998; Naveilhan et al., 1998).

5.2 ROLE OF GFRα2/NRTN SIGNALING DURING SENSORY NEURON ONTOGENY

During DRG neurogenesis, three distinct populations of Ret-expressing (Ret+) neurons are generated. The early Ret+ neurons begin to express Ret on, or before E10.5. These neurons are TrkA-negative and exclusively express GFRα2 (Luo et al., 2009). A second, intermediate population of Ret+ neurons emerges from TrkA-positive precursors, beginning at approximately E13.5 and expresses GFRα1. The third population of Ret+ neurons is the non-peptidergic sensory neurons. These neurons also originate from TrkA-positive precursors, express Ret at E15.5 and GFRα2 from P0 onward (Luo et al., 2007; Luo et al., 2009). Thus, two distinct

populations of GFRα2-expressing neurons are generated during development. The early (E10.5) Ret/GFRα2-positive neurons have relatively large somata (287 ± 102 μm^2) and almost none express CGRP or bind IB_4. These neurons are thought to be rapid adapting mechanoreceptors that form Meissner corpuscles, Pacinian corpuscles, and longitudinal lanceolate endings (Luo et al., 2009). In NRTN-OE mice, we found an increase in the size of large diameter myelinated axons in the saphenous nerve. The affected axon might represent hypertrophy of early Ret/GFRα2-positive low threshold mechanosensitive neurons. The second population of GFRα2-positive neurons is detectable at P0 and originates from TrkA-positive precursors. At E14.5, the majority of Ret-negative neurons expressed TrkA and Runx1, and require NGF for survival. Persistent Runx1 expression is thought to suppress TrkA expression and activate Ret expression in a subpopulation of DRG neurons. Some of these Ret-positive/IB_4-binding neurons become GDNF-dependent (Molliver et al., 1997; Chen et al., 2006; Luo et al., 2007) and express GFRα1 (Luo et al., 2007). However, the expression of both GFRα1 and α2 is relatively sparse as late as E16 (Baudet et al), a time after the first wave of programmed cell death that decreases the sensory neuron population by up to 50% (Davies, 1996). Thus, it is not surprising that sensory neuron loss in GDNF KO mice is moderate (23%) compared to the loss seen following deletion of growth factors in the neurotrophin family (55-80% (NT-3), 30% (BDNF), 70% (NGF)) whose receptors are expressed early and robustly during embryogenesis (Mendell, 1996). Similarly, there is no obvious cell loss in NRTN KO mice despite a reduction in the number of GFRα2-immunoreactive neurons (Heuckeroth et al., 1999). Overexpression of GDNF, NRTN or ARTN also produce moderate effects; overexpression of GDNF, ARTN or NRTN in the skin increased the total number of sensory neurons 27%, 21%, 0%, respectively (Zwick et al., 2002; Elitt et al., 2006).

The small effect on sensory neuron number seen following genetic deletion of GFL members suggests that these growth factors do not contribute to cell survival as regulated by developmental programmed cell death. However, these factors can have significant survival effects following injury, especially if this injury occurs during the neonatal period. Baudet et al., (2000) found that culturing sensory neurons from neonatal mice between the ages of P0 to P15 results in the death of up to 90% of all plated neurons. However, addition of GDNF, NRTN or ARTN can prevent cell death by up to 60% when applied individually and up to 80% when applied in combination. The results presented in this dissertation show that the ability to rescue adult dissociated neurons from cell death is retained for GDNF and ARTN, though fewer adult neurons die as a result of dissociation in the absence of growth factor supplementation. In contrast, NRTN, which can rescue up to 50% of P15 sensory neurons, has no effect on the survival of adult sensory neurons in culture.

In summary, the function of GFLs changes during development. Although NRTN is not required for the survival of GFRα2-positive neurons, it regulates the phenotype of GFRα2-positive neurons. Compared to neurotrophins that regulate neuron survival during embryogenesis, the effect of GFLs, if there is one, occurs in the postnatal period.

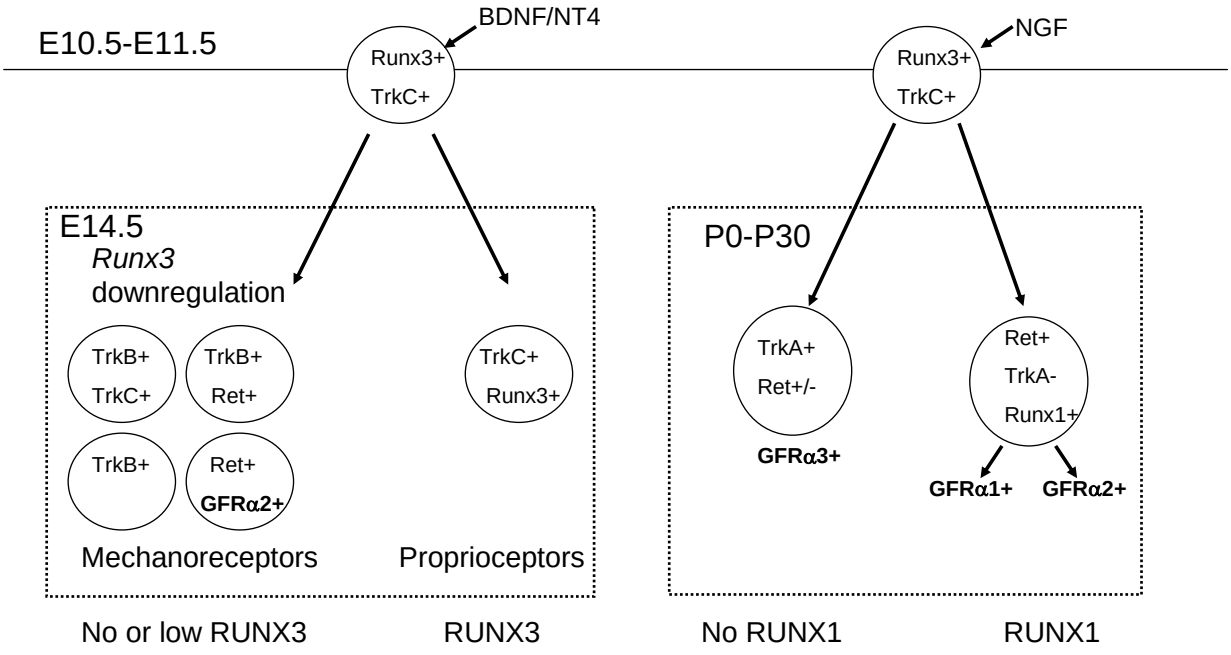


Figure 32. Development of GFRα2-positive neurons.

During development, two distinct populations of GFRα2-expressing neurons are generated. One population expresses GFRα2 as early as E10.5 (Luo et al., 2009). These early Ret/GFRα2-positive neurons originate from $Runx3^{+}/TrkC^{+}$ precursor neurons, have relatively large somata ($287 \pm 102\ \mu m^2$) and almost none express CGRP or bind IB_4. The second population of GFRα2-positive neurons is detectable at P0 and originates from $Runx1^{+}/TrkA^{+}$ precursors. These neurons are small in diameter (100-250 μm^2), and likely represent $Ret^{+}/IB4^{+}$ polymodal nociceptors (Marmigere and Ernfors, 2007).

5.3 ROLE OF GFRα SIGNALING IN SENSORY NEURON PLASTICITY

The results presented here, together with previous studies show that the majority of GFRα1 and α2 neurons are non-peptidergic C-fibers (i.e., do not express CGRP or SP), whereas virtually all GFRα3 expressing neurons are peptidergic, express the heat-sensitive channel TRPV1 and 80% express TrkA (the tyrosine kinase receptor for NGF) (Bennett et al., 2000; Orozco et al., 2001; Airaksinen and Saarma, 2002; Airaksinen et al., 2006; Albers et al., 2006b; Elitt et al., 2006; Lindfors et al., 2006).

After peripheral nerve injury, GFRα receptor expression is differentially regulated. We find that after saphenous nerve axotomy, GFRα2 expression in injured neurons is significantly downregulated, whereas expression of GFRα1 and GFRα3 increase. We also found that in 39% of GFRα2-expressing neurons, the loss of detectable GFRα2 is replaced by GFRα3 expression and that this may be regulated by Runx1, a transcription factor critical for differentiation of nociceptor subtypes (Chen et al., 2006). The change of GFRα receptors in sensory neurons may be related to the change of GDNF family growth factors in the peripheral targets of these neurons. After saphenous nerve injury, mRNA level of GDNF and ARTN are increased, whereas NRTN level is not changed in hindpaw skin (Jankowski et al., 2009). This suggests that target-derived growth factor production, as well as sensory neuron expression of the corresponding receptors, is responsive to the functional integrity of the afferent axon. That is, upon injury both the target and neuron exhibit abnormal expression of growth factors and receptors. How these changes contribute to regeneration of peripheral nerves, or injury pathology, is not clear. The switch of sensory neuron phenotype with more neurons expressing GFRα3 after nerve axotomy could explain the ability of ARTN to improve the regeneration of neurons that do not normally

express the GFRα3 receptor (Wang et al., 2008). In fact, in the Wang et al. study (2008) regeneration of central sensory fibers is seen, a phenomena that does not occur in the absence of experimental manipulation. The downregulation of GFRα2 expression combined with up regulation of GFRα3 after nerve injury suggests that either GFRα2 signaling is less effective than GFRα3 at stimulating regeneration, or that GFRα2 signaling might actually repress regeneration, for example by stabilizing existing connections in NRTN-responsive neurons. This hypothesis could be tested by forced GFRα2 expression after axotomy combined with addition of NRTN.

5.4 NRTN REGULATION OF THERMAL AND MECHANICAL RESPONSIVENESS

Although NRTN does not appear to be required for sensory neuron survival, it is required for maintenance and proper function of GFRα2 neurons. In NRNT KO mice, the GFRα2+ DRG neurons are hypotrophic, exhibiting smaller somata and axons (Heuckeroth et al., 1999). GFRα2 KO mice also display peripheral target innervation defects, primarily for nonpeptidergic sensory neurons (Lindfors et al., 2006). Our results show that overexpression of NRTN induces hypertrophy of GFRα2 neurons and hyperinnervation of the skin by nonpeptidergic fibers. NRTN also upregulated cold sensitive channels TRPM8, TRPA1, the mechanical sensitive channel ASIC2a, and the ATP receptor $P2X_3$. Interestingly, all of these channels in addition to GFRα2 are regulated during development by Runx1. This suggests the potential for reciprocal regulation of Runx1 via GFRα2/Ret-signaling. That Runx1 would be regulated by Ret signaling would not be novel in that data from the present experiments and others have shown that GDNF

(Wang et al., 2003; Averill et al., 2004) and ARTN (present studies) can regulate the expression of ATF3, another transcription factor, in injured sensory neurons.

GDNF, like NRTN, also increased expression of ASIC2 (in this case both the 2a and 2b isoforms). It had been proposed previously that ASIC channels contribute to mechanical sensitivity (Price et al., 2000; Garcia-Anoveros et al., 2001; Welsh et al., 2002). The increase in ASIC2a and 2b in GDNF-OE mice was correlated with a decrease in mechanical thresholds in C-polymodal nociceptors in the *ex vivo* physiology preparation (Albers et al., 2006), although these mice exhibited no change in mechanical sensitivity in behavioral tests (Zwick et al., 2002). In contrast, the increase in ASIC2a in the NRTN-OE mice was correlated with an increase in mechanical sensitivity in behavioral tests. When examined at the physiological level, NRTN-OE mice exhibited more mechanically sensitive C-fibers, but these had such high threshold they were unlikely to contribute to observed changes in behavior.

Why NRTN-OE mice exhibit behavioral, but not physiological mechanical hypersensitivity is unclear. It is possible the *ex vivo* preparation did not accurately reflect the threshold of the additional mechanically sensitive C-fibers. An alternative explanation is that these fibers do not contribute to behavioral sensitivity and that other mechanisms are involved. For example, the electron microscopic analysis of the saphenous nerve showed that the entire size distribution of C-fibers was shifted to the right, indicating that the majority of cutaneous afferents had larger axons. The increase in axon size was consistent with the increase in soma diameters and was likely due to the increase in terminal branching of NRTN-responsive afferents that include mechanically-sensitive C-fibers. The increase in axonal diameter was correlated with increased conduction velocity in all classes of C-fibers except for C-cold fibers (and this may have been due to the small sample size of these fibers). The increase in conduction velocity by

itself could account for increased behavioral responses if it resulted in greater temporal summation of afferent input to second order dorsal horn neurons. Thus, the increase in behavioral responses could be independent of any change in expression of a particular channel or receptor, or even an increase in the number of mechanically sensitive afferents.

NRTN-OE mice were also more sensitive to cool temperatures and menthol. Correlated with these changes was an increase in TRPM8 mRNA and protein in sensory neurons. TRPM8 is the only known menthol receptor and has been implicated in detection of cool temperatures (McKemy et al., 2002; Bautista et al., 2007; Colburn et al., 2007). Thus, the increase in sensitivity in NRTN-OE mice can be explained simply by increased expression of TRPM8. However, in addition to these biochemical changes we observed the appearance of TRPM8 staining in a population of neurons with medium size somata and these neurons were not TRPM8-immunoreactive in WT mice. Whether these neurons play a role in either cool or menthol hypersensitivity will require additional experiments.

NRTN-OE mice also exhibited behavioral thermal hyposensitivity on a -20^{o}C ice block as well as at 41^{o}C. The reason for these behavioral changes is not clear. TRPA1 has been suggested to be involved in the detection of noxious cold (Story et al., 2003; Bautista et al., 2006). TRPA1 mRNA is increased in the NRTN-OE mice which would be consistent with an increase in cold sensitivity and not the observed decrease. Moreover, the increase in TRPA1 was not associated with an increase in sensitivity at other noxious cold temperature in the two-temperature choice test where TRPA1 is reported to be activated. There were no changes in gene expression that could account for the decreased behavioral sensitivity that was seen at 40^{o}C. As we did not measure protein in these studies it is possible that there were changes in protein translation that was not detected by looking at mRNA levels. Other genes such as P2Y2 and

P2Y1 could potentially contribute to noxious heat detection (Molliver et al., ; Malin et al., 2008). Neither of these was changed. Thus, additional experiments will have to be conducted to determine the cause of this hyposensitivity.

5.5 CONCLUSION AND FUTURE DIRECTIONS

These studies confirm that NRTN acts on a specific subset of cutaneous afferents where it modulates various aspects of anatomy and function that have consequences for behavioral responses. NRTN appears to play a central role during development, and may have its greatest effects during the neonatal period when cutaneous afferents are completing the differentiation process. Because the majority of NRTN-responsive neurons are polymodal C-fibers, it is not surprising that overexpression of NRTN altered the response properties of these neurons to multiple stimuli including cold, cooling and mechanical. Overexpression of NRTN may also increase the receptor field of these neurons. We could use the ex vivo preparation to map the receptor field of individual neuron in NRTN-OE DRG to test this hypothesis.

The *in vitro* studies indicate that NRTN signaling may be unique in that it does not appear to be able to alter changes associated with injury, as has been seen for other sensory neuron growth factors including other members of the GFL family. In fact, the data presented here suggests that one of the first actions of damaged sensory neurons is the down-regulation of GFRα2 and up-regulation of a different GFRα both *in vitro* an *in vivo*. The down-regulation of NRTN and GFRα2 after nerve injury *in vivo* suggested that these might be the signals to initiate the regeneration. One experiment we could do in the future is to intrathecal infusion of NRTN

after peripheral nerve injury and exam whether increased NRTN could slow the progress of regeneration.

The changes seen in the *in vitro* studies also have important implications for the interpretation of data from experiments using cultured sensory neurons. Laboratories including ours have used cultured neurons as a model for intact afferents. The results reported here demonstrate clearly that dissociation of neurons immediately alters the phenotype of these cells so that they no longer represent the same populations of afferents seen *in vivo*. This is particularly true of NRTN-responsive GFRα2-positive neurons that rapidly begin to alter their gene expression profile to GFRα3-positive sensory neurons. We suggested that the switch of phenotype might under regulation of transcription factor Runx1. This hypothesis could be tested by forced expression of Runx1 *in vitro* and test whether Runx1 could reverse the loss of GFRα2 and the increase of GFRα3 expression in DRG neurons. In conclusion, cultured sensory neurons are probably best used to model injured sensory neurons and to use them for other purposes will require the use of careful controls to incorporate the types of changes described here.

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