MOLECULAR MECHANISMS OF AGING IN THE PERIPHERAL NOCICEPTIVE SYSTEM

by

Shuying Wang

MD, Shandong University, 1995

MS, Shandong University, 1997

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Shuying Wang

It was defended on

December 14, 2006

and approved by

J. Patrick Card, Ph.D., Neuroscience and Psychiatry

Brian M. Davis, Ph.D., Medicine and Neurobiology

H. Richard Koerber, Ph.D., Neurobiology

Carl F. Lagenaur, Ph.D., Neurobiology

Stephen W. Scheff, Ph.D., University of Kentucky

Dissertation Director: Kathryn M. Albers, Ph.D., Medicine and Neurobiology

MOLECULAR MECHANISMS OF AGING IN THE PERIPHERAL NOCICEPTIVE SYSTEM

Shuying Wang MD, PhD

University of Pittsburgh, 2006

Decreased pain sensitivity during aging is common in humans and animals and is thought to reflect changes in anatomical, functional and cellular properties of the peripheral nervous system (PNS). We hypothesized that a reduction in neurotrophic growth factor and ion channel expression led to some of these age-associated changes in the PNS. To test this, a detailed comparative study was made of 6~8 week-, 16 month- and 2 year-old Blk6 male mice obtained from the NIA mouse colony. Behavioral assays showed aged mice had decreased sensitivity to noxious heat and impaired inflammation-induced thermal hyperalgesia. To understand the basis for this change we examined expression of the growth factor artemin, its receptor GFRα3 and TRPV1, an ion channel expressed by 95~99% of GFRα3-positive sensory neurons. TRPV1 is of significance since it is required for transmission of thermal hyperalgesia following tissue inflammation. Assays showed a reduction in TRPV1 mRNA and protein in the PNS of aged mice that correlated with a decrease in expression of the artemin receptor GFRα3. CFA-induced inflammation also increased artemin expression in the skin but decreased expression of GFRα3 mRNA in the dorsal root ganglia (DRG) of both young and old mice. The decrease in GFRα3 was greater in aged mice, suggesting GFRα3 signaling following CFA is also reduced and that the response properties of GFRα3-positive sensory neurons that express TRPV1 are diminished. Calcium imaging of isolated primary neurons grown with NGF was therefore used to test the *in*

vitro effects of artemin on TRPV1 activation in young and old neurons. Artemin potentiated TRPV1 activation by capsaicin in young and old neurons, but the amplitude of capsaicin responses in young neurons was decreased with long-term exposure to artemin. In studies using microarrays and RT-PCR, inflammation-associated genes such as interleukin 6 (IL-6) were found elevated in sensory ganglia of aged mice. This ongoing inflammatory state may increase the inflammatory tone of the system and contribute to changes in response properties and sensitivity of sensory neurons in the aging PNS. Thus, the reduced sensitivity to inflammatory pain in aged animals reflects a combination of changes in anatomical, physiologic and immune response properties.

ACKNOWLEDGEMENTS

First I deeply thank my mentor Dr. Kathryn Albers for her tremendous support throughout my PhD training from the University of Kentucky to the University of Pittsburgh. Without her dedication to my academic and personal development, without her guidance and encouragement, it would be impossible to complete this challenging dissertation. To me Dr. Albers is not only a great mentor but also a colleague in science and a friend in life.

I especially thank Dr. Brian Davis and Dr. Sacha Malin for their invaluable academic advice and technical assistance. Without their generous help and support I would not have completed this dissertation in time. I am also very grateful to the members of my dissertation committee, Dr. Richard Koerber, Dr. Patrick Card, Dr. Brian Davis, Dr. Carl Lagenaur and my outside examiner Dr. Stephen Scheff, for their commitment to my dissertation project and academic development.

Special thanks go to the members of the Albers and Davis Labs, Pam Cornuet, Dr. Chris Elitt, Beth Knapick, Dr. Sacha Malin, Dr. Derek Molliver, and Mela Poonacha for sharing their skills, experience and knowledge with me. In particular, I thank Jessica Lindsay, Nozomi Sakai, Chris Sullivan and Dr. Melissa Zwick for their specific technical support in my dissertation project.

I was lucky to be in the great training program of the CNUP. This program not only trained me how to think creatively as well as critically but will also shape the way I do science in the future. I appreciate the support and assistance from many faculty members, graduate students, postdocs and office staff, especially Patti Argenzio, within the program.

Finally I extend my gratitude to my family. They are my strength and inspiration. Without their constant support behind me this dissertation would not have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. OVERVIEW

PAIN SIGNALING IN THE PERIPHERAL NOCICEPTIVE SYSTEM

Pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage", according to the International Association for the Study of Pain (IASP). Under normal conditions the experience of pain has an important protective role. In pathological conditions, the sensation of pain also has a protective response and functions to prevent further damage to the already injured tissue. However some pathological conditions, such as chronic joint inflammation or nerve injury, result in persistent and recurrent pain that can severely affect daily activities if left untreated. Persistent pain is more common in older people who are at a much higher risk for such pain-related morbidity. Although the relative risk and the nature of pain in elderly people is influenced by age-related painful diseases, co-morbid illness, and psychological changes associated with aging, accumulating evidence suggests that pain severity is also influenced by age-related changes in the structure and functional properties of the peripheral nociceptive system (PNS). To identify how changes in the PNS might relate to pain transmission and sensitivity with aging, we compared the cutaneous sensory system of young and old mice at the cellular and molecular level. The overall goal was to determine if age-related changes occurred in neurotrophic growth factor expression, ion channel expression or response properties of sensory neurons.

As a sensory organ, skin contains a dense network of highly specialized primary afferent nerve fibers whose cell bodies are found in the dorsal root ganglia (DRG) and trigeminal ganglia (TG) (Iggo and Andres, 1982). The terminals of the afferent nerve fibers form receptors that detect and transmit thermal, mechanical and chemical information to the central nervous system (CNS). Generally speaking, spinal afferent fibers from DRG neurons enter and form synapses

1

with neurons in the dorsal horn of the spinal cord, and their axons cross to the contralateral side and ascend to synapse on neurons in the thalamus. Thalamic neurons send fibers to the cortex where conscious sensation occurs (McHugh and McHugh, 2000). The first physiological step of complex pain processing, i.e., nociception, involves the transmission of a noxious signal by a subset of primary sensory neurons called nociceptors (Dubner and Bennett, 1983; Besson and Chaouch, 1987; Millan, 1999). Nociceptors are selectively activated by diverse noxious stimuli, and anatomically and functionally distinct from non-nociceptive neurons that detect innocuous stimuli (Caterina and Julius, 1999). Nociceptors set specific response thresholds to distinguish between noxious and innocuous events and reset these thresholds following injury to sensitize the system and prevent further injury (Julius and McCleskey, 2006).

1.1 FUNCTIONAL PROPERTIES OF NOCICEPTORS

Cutaneous nociceptors can be divided into two main groups based on anatomical and functional properties: unmyelinated C-fiber nociceptors with slower conduction velocity and myelinated A-fiber nociceptors with faster conduction velocity (Dubner and Bennett, 1983; Djouhri and Lawson, 2004). There are two types of A-fiber nociceptors: type I and type II (Treede et al., 1998). Type I fibers are present in both hairy and glabrous skin while type II are only in hairy skin. When a noxious stimulus is applied to the skin, activated cutaneous nociceptors generate and transmit an electrical signal (action potential, AP) along A-fibers and/or C-fibers to the dorsal horn of the spinal cord, and elicit fast sharp pain and/or slow dull pain (Julius and Basbaum, 2001). According to their responses to different modalities of noxious stimulation, nociceptors can be further classified into thermal, mechanical and chemical nociceptors. Most are polymodal nociceptors that respond to thermal, mechanical and chemical stimuli, whereas other nociceptors are activated only by a subset of these modalities (Caterina and Julius, 1999).

1.1.1 Thermal nociception

Responses of nociceptors to heat stimuli have been studied in detail and a wide range of heat thresholds from 38°C to 53°C has been reported (Spray, 1986). Thermal modeling studies have shown that the heat threshold of nociceptors depends on the temperature at the depth of the receptors. The transduction of heat stimuli (conversion of heat energy to action potentials) occurs at different skin depths for different nociceptors and the supra-threshold responses of nociceptors vary directly with the rate of temperature increase (Tillman et al., 1995b, 1995a). When the rate of temperature increases very slowly or when stimulus duration is very long, the heat threshold of most C-fiber nociceptors and type II A-fiber nociceptors is between 39°C and 41°C (Tillman et al., 1995a), and type I A-fiber nociceptors is between 40~50°C (Treede et al., 1998; Meyer et al., 2006). Further studies have found that human sensation of pain to stimuli over the range of 41~49°C correlates very well with the activity of C-fiber nociceptors (Meyer et al., 2006) and the thermal threshold of type II A-fiber nociceptors in hairy skin is near the threshold temperature for initiation of pain sensation (Beitel et al., 1977; Treede et al., 1998). These observations indicate a role of nociceptors in heat-induced pain sensation.

1.1.2 Mechanical nociception

Mechanical responses of nociceptors depend on the features of a mechanical stimulus and the type of nociceptor activated. Peak response occurs at the onset of the stimulus followed by slow adaptation to stimuli. The response of A-fiber nociceptors is greater than C-fiber nociceptors (Slugg et al., 2000). In general the activity of nociceptors increases with pressure and force except that the response of C-fiber nociceptors saturates at higher force level (Slugg et al., 2000). A-fiber nociceptors are thought to be responsible for transmission of sharp pain induced by punctate mechanical stimuli since the reaction time to perceive sharp pain is short and the stimulus-response function of A-fiber nociceptors is comparable with the pain ratings of human subjects (Magerl et al., 2001). When long-duration mechanical stimuli are applied, the pain increases throughout the stimulus. Certain C-fiber nociceptors that are normally insensitive to mechanical stimuli develop a response to prolonged mechanical stimulation, and are thought to

signal pain associated with tonic pressure since selective block of A-fiber nociceptors rarely affects the tonic pain (Andrew and Greenspan, 1999; Schmidt et al., 2000).

1.1.3 Chemical nociception

A variety of chemical agents, including endogenous inflammatory mediators (e.g. acid) and exogenous agents (e.g. capsaicin and formalin) can activate nociceptors and produce pain (Reichling and Levine, 1999). Intradermal injection of capsaicin induces a long-lasting vigorous response in certain A- and C-fiber nociceptors and intense pain that lasts for several minutes, suggesting these fibers are responsible for capsaicin-induced pain (Schmelz et al., 2000; Ringkamp et al., 2001). A channel protein expressed in nociceptors, TRPV1, has been found to mediate the noxious effects of capsaicin (Caterina et al., 1997). Heat and protons can also activate TRPV1, indicating that neurons expressing TRPV1 may be polymodal nociceptors. It should be mentioned that most chemical agents probably cause tissue injury and hence induce pain, which is particularly true for inflammation-induced pain.

A common feature found in nociceptors that respond to thermal, mechanical and/or chemical (capsaicin) stimuli, is time-dependent desensitization by repeated stimuli, which is a reduction of the response to the second of two identical stimuli compared with the response to the first one, and in turn results in a reduction in pain intensity following repeated stimuli (Slugg et al., 2000; Witting et al., 2000; Peng et al., 2003). The mechanism for capsaicin-induced desensitization has been intensively studied. In addition, nociceptors not only encode the intensity and modality but also encode spatial localization of noxious cutaneous stimuli, and actual pain thresholds are higher in vivo than the thresholds for activation of individual nociceptors, indicating involvement of central mechanisms in regulation of nociception (Millan, 1999).

1.2 MOLECULAR PROPERTIES OF NOCICEPTORS

Over the past two decades rapid progress has been made in defining the molecular and cellular mechanisms of cutaneous nociception. Physiological function of nociceptors depends on distinct expression of receptors and ion channels that can be activated by noxious stimuli. These nociceptive receptors and ion channels detect specific physical or chemical stimuli and induce membrane depolarization. Voltage-gated ion channels are in turn activated and produce APs, and result in neurotransmitter release at spinal synapses. Among these receptors and ion channels, transient receptor potential ion channel (TRP)-related TRPVs, acid-sensing ion channels (ASICs) and purinergic ionotropic receptors (P2X2 and P2X3) have been well studied and recognized as molecular detectors of noxious thermal, mechanical or chemical stimuli (Caterina and Julius, 1999; Julius and Basbaum, 2001).

1.2.1 Molecular detectors of noxious stimuli

As mentioned before some nociceptors have a lower thermal threshold than others. In vitro studies also show that about 45% of DRG neurons respond to heat with a threshold of ~ 42°C while 5~10% respond with a higher threshold of ~ 51°C (Nagy and Rang, 1999). The difference of nociceptors in thermal threshold is determined by specific expression of heat-sensitive ion channels, especially TRPVs. TRPV1 is predominantly expressed in unmyelinated C-fiber nociceptors and has a thermal activation threshold of ~ 43°C, indicating involvement of TRPV1 in thermal response of nociceptors with lower threshold (Caterina et al., 1997; Tominaga et al., 1998; Michael and Priestley, 1999; Caterina et al., 2000). TRPV2, mainly present in myelinated A-fiber nociceptors, has a thermal threshold of ~ 52°C and is thought to mediate high threshold heat responses (Caterina et al., 1999). TRPV3 and TRPV4 have been found to respond to heat with thermal threshold between 31 and 39°C, but their expression and function in thermal nociceptors is controversial (Guler et al., 2002; Peier et al., 2002; Smith et al., 2002; Watanabe et al., 2002; Xu et al., 2002). TRPV4 can also be activated by changes in osmolarity suggesting that TRPV4 might be also involved in mechanosensation (Liedtke et al., 2000; Strotmann et al., 2000; Suzuki et al., 2003b).

Despite the fact that mechanosensitive channels such as ASIC1, ASIC2 and TRPV4 are present in nociceptors (Alvarez de la Rosa et al., 2002), molecular detectors of mechanical stress remain elusive since deletion of ASICs or TRPV4 genes only produces subtle changes in mechanosensation, osmoregulation or nociception (Price et al., 2000; Price et al., 2001; Suzuki et al., 2003a). Though some chemical agents like capsaicin can directly produce pain via activation of TRPV1, most noxious chemical stimuli are endogenously released following tissue injury. Acidosis is a common consequence of injuries associated with inflammation and ischemia. TRPV1 and ASICs are thought to mediate acid-induced pain since both can be activated by protons as well as expressed in acid-responsive nociceptors (Caterina et al., 1997; Caterina et al., 2000; Price et al., 2000; Price et al., 2001; Alvarez de la Rosa et al., 2002). Tissue injury also results in ATP release from damaged cells. ATP can directly activate the purinergic ionotropic receptors P2X2 and P2X3, which are preferentially expressed in nociceptors, and induce the sensation of pain (Chen et al., 1995; Lewis et al., 1995; Cook et al., 1997; North, 2004).

1.2.2 Molecular signal transducers

Once the molecular detectors in nociceptors are activated by noxious stimuli, voltage-gated ion channels are opened by membrane depolarization and action potentials are produced and propagated. Action potentials of nociceptors are remarkably long in duration and relatively slow at firing rate (Koltzenburg et al., 1997; Djouhri et al., 1998). To date many types of voltage-gated sodium, potassium and calcium channels have been identified in primary sensory neurons, but only some are specifically or preferentially expressed in nociceptors, which might be the molecular basis for the distinct excitability of nociceptors. Two of them are tetrodotoxin (TTX)-resistant voltage-gated sodium channels Nav1.8 and Nav1.9 (Akopian et al., 1999; Amaya et al., 2000; Fang et al., 2002; Djouhri et al., 2003). Slow inactivation of these two channels leads to long duration of APs in nociceptors (Djouhri et al., 2003). Voltage-gated calcium channels (VGCC), such as N-type voltage-gated calcium channel Cav2.2, also shapes the prolonged shoulder of APs in nociceptors (Blair and Bean, 2002). Studies have shown that Cav2.2 carries the bulk of calcium current in sensory neurons and the fraction is relatively higher in nociceptors than in others, and neurotransmission at the first synapse in the nociceptive pathway mainly relies on Cav2.2 (Mintz et al., 1992; Gruner and Silva, 1994; Cardenas et al., 1995; Rusin and

Moises, 1995; Kim et al., 2001). A splicing variant of Cav2.2, e37a, has been reported to be preferentially expressed in capsaicin-responsive neurons (Bell et al., 2004). These findings indicate relative specificity of involvement of Cav2.2 in nociception.

1.2.3 Molecular properties determine nociceptor function

The molecular detectors of noxious stimuli plus voltage-gated ion channels for signal propagation determine sensitivity and activity of nociceptors. Changes in expression and/or function of these nociceptive channels and receptors may result in functional changes of nociceptors. It has been reported that increased expression or activity of Nav1.8 and Nav1.9 results in increased sodium conductance, decreased AP threshold, and increased activation rate and firing rate, and in turn causes hyper-excitability of sensory neurons (Gold, 1999; Waxman et al., 1999). Other studies also have shown that in control DRG neurons, ATP or protons only induce sub-threshold membrane depolarization while in inflamed neurons with increased expression of ASIC3 or P2X2/3 the same pH drop or the same amount of ATP evokes supra-threshold depolarization and triggers action potentials, indicating that up-regulation of detector proteins may contribute to hypersensitivity and hyper-excitability of sensory neurons (Mamet et al., 2002; Xu and Huang, 2002). *In vivo* inflammation not only causes persistent pain but also hyperalgesia, an increased response to a stimulus that is normally painful, and allodynia, a painful response due to a stimulus which does not normally provoke pain. The molecular basis for increased sensitivity and activity of nociceptors during inflammation is consistent with the molecular mechanism of nociception discussed above. In general, inflammation increases expression and/or function of nociceptive-related channels, thus decreasing activation thresholds and increasing AP production in nociceptors resulting in pain (Okuse et al., 1997; Gold, 1999; Waxman, 1999; Waxman et al., 1999; Voilley et al., 2001; Ji et al., 2002; Xu and Huang, 2002). This model is also consistent with the finding that deletion of these channels either decreases or slows occurrence of inflammatory pain (Barclay et al., 2002; Walker et al., 2003). During inflammation channel expression and/or function in nociceptors can be modulated by nerve growth factor (NGF). NGF binds to the TrkA receptor tyrosine kinase (RTK) receptor, and activates second message signaling pathways that are thought to regulate gene expression or modification, i.e. phosphorylation, of channel proteins (Julius and Basbaum, 2001).

1.3 REGULATION OF NOCICEPTOR FUNCTION

Neurotrophic factors regulate the long-term survival, growth and differentiated function of distinct populations of sensory neurons. Multiple types of cells can produce neurotrophic factors, including those in target tissues such as the skin, supporting glial cells in sensory ganglia such as the satellite and Schwann cells and immune cells that are found in the skin and ganglia, e.g., macrophages (Batchelor et al., 1999). Two families of neurotrophic factors, the neurotrophin family, comprised of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), and the glial cell line-derived neurotrophic factor (GDNF) family, have been found to be required for development, maintenance and function of sensory neurons. The action of neurotrophic factors depends on their binding to transmembrane receptors. NGF binds to the receptor p75 and the receptor tyrosine kinase TrkA (Barbacid, 1995; Chao and Hempstead, 1995). GDNF binds to the Ret receptor tyrosine kinase and to a glycosyl-phosphatidyl inositol (GPI)-anchored receptor GFRα (Saarma and Sariola, 1999). Based on the requirement for trophic factors, nociceptors can be divided into two groups: NGF-dependent nociceptors, which express peptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), and GDNF-dependent nociceptors, which are peptide poor and bind the plant isolectin B4 (IB4) (Snider and McMahon, 1998). In adult rodents about 40% of DRG neurons are NGF-dependent and 30% are GDNF-dependent (Molliver et al., 1997). Although the two groups of nociceptors may have distinct roles in pain sensation, neurons in each group respond to capsaicin and heat and in rat, many express TRPV1 (Tominaga et al., 1998; Michael and Priestley, 1999).

1.3.1 NGF

The important role of NGF in development and differentiation of nociceptors has been well established. NGF also regulates nociceptor function in the adult (Bennett, 2001). In vitro studies have shown that NGF regulates gene expression of SP and CGRP in cultured adult DRG neurons as well as chemical sensitivity of nociceptors to capsaicin and protons (Winter et al., 1988; Lindsay and Harmar, 1989; Bevan and Winter, 1995). *In vivo* studies have found that subcutaneous administration of NGF sensitizes nociceptors to thermal and chemical stimuli and

causes hyperalgesia (Lewin et al., 1993). Further studies indicate that local infusion of anti-TrkA IgG, an NGF signaling antagonist, decreases thermal and chemical sensitivity of nociceptors and leads to thermal hypoalgesia, suggesting TrkA is required for regulation of nociceptor function by NGF (McMahon et al., 1995). By binding and activating TrkA-mediated signaling pathways NGF regulates not only expression but also post-translational modification of receptors and ion channels involved in nociception such as TRPV1 and Nav1.8 (Gold et al., 1998; Kerr et al., 2001; Bonnington and McNaughton, 2003; Zhang et al., 2005). Increased expression and specific phosphorylation of TRPV1 and Nav1.8 by NGF are thought to contribute to NGF-induced sensitization of nociceptors (Julius and Basbaum, 2001; Kerr et al., 2001; Ji et al., 2002).

It is clear now that NGF can act as an inflammatory mediator and is essential for inflammatory hyperalgesia (Mendell et al., 1999; Bennett, 2001). NGF has been found to increase in inflamed tissue, and this increase may be secondary to cytokine production, such as IL-1 and TNFα (Woolf et al., 1994; Safieh-Garabedian et al., 1995; Woolf et al., 1997). Anti-NGF or anti-TrkA treatment diminishes the hyperalgesia (Woolf et al., 1994; McMahon et al., 1995). During inflammation nociceptors display increased thermal and mechanical sensitivity, which can also be prevented by anti-NGF treatment. As mentioned above, NGF can act directly on nociceptors by modifying channel proteins like TRPV1 to induce hyperalgesia. In addition NGF can also up-regulate channel protein expression, which may contribute to long-term hyperalgesia (Koltzenburg et al., 1999). Another possibility is that NGF causes cytokine release from mast cells, acts on sympathetic efferents, or activates other signaling pathways, which can also be involved in hyperalgesia (Bennett, 2001).

1.3.2 GDNF family ligands

The role of GDNF and GDNF family ligands (GFLs) in functional regulation of nociceptors in the adult is less understood and more controversial. The GDNF growth factor family consists of GDNF, neurturin (NTN), artemin (ART) and persephin (PSP), which preferentially bind to GFRα1, GFRα2, GFRα3 and GFRα4, respectively. The GFRs in conjunction with the Ret form a receptor complex (Saarma and Sariola, 1999). GDNF, NTN and ART support the survival and differentiation of subpopulations of cutaneous sensory neurons *in vitro* (Baudet et al., 2000).

Disruption of GDNF results in a 23% decrease of total neurons in DRG and selective loss of GFRα1-expressing neurons in TG at P0 (Moore et al., 1996). However, disruption of GFRα1 has no effect on the survival of sensory neurons in either DRG or TG (Cacalano et al., 1998). Deletion of NTN causes selective loss of GFRα2-expressing neurons in TG and knockout of GFRα2 decreases peripheral innervation without affecting the number of DRG neurons (Heuckeroth et al., 1999; Rossi et al., 1999; Stucky et al., 2002; Lindfors et al., 2006). No deficiency has been reported in sensory ganglia of ART or GFRα3-knockout mice though GFRα3 is predominantly expressed in peptidergic nociceptive sensory neurons (Nishino et al., 1999; Orozco et al., 2001; Honma et al., 2002). The lack of sensory neuron abnormalities in these knockout studies may be due to crosstalk among GDNF, NTN and ART signaling pathways (Sariola and Saarma, 2003).

The influence of GDNF on nociception may vary between rats and mice due to distinct difference in the expression pattern of TRPV1 in GDNF-dependent nociceptors. In rats more than half of GDNF-dependent nociceptors express TRPV1 whereas in mice only 2~5% are TRPV1-positive (Guo et al., 1999; Michael and Priestley, 1999; Zwick et al., 2002). Not surprising, overexpression of GDNF in mouse skin does not affect either heat thresholds of GDNF-dependent nociceptors or the percentage of TRPV1-expressing neurons in sensory ganglia since few mouse GDNF-dependent nociceptors express TRPV1. But overexpression of GDNF increases the percentage of GDNF-dependent, IB4-positive neurons in DRG and decreases mechanical thresholds of these neurons (Albers et al., 2006). Moreover, GDNF overexpression results in increased P2X3-positive cutaneous nerve fibers, which are highly expressed in GDNF-dependent nociceptors (Zwick et al., 2002).

Neurturin is also thought to support a subpopulation of neurons that have nociceptor properties. Approximately 50% of GFRα2–expressing neurons respond to noxious heat and deletion of the GFRα2 gene decreases the percentage of neurons with large heat-evoked response and increases the percentage of neurons with small or no heat response. Loss in neurons with large heat responses may contribute to a deficit in heat transduction (Stucky et al., 2002). These studies support a role for GDNF family members in nociception. However, neither overexpression nor deletion of GDNF/GFRα changes behavioral responses to noxious heat and

mechanical stimulation (Zwick et al., 2002; Lindfors et al., 2006), suggesting that *in vivo*, other mechanisms must be involved in nociception.

Following inflammation in the rat, anti-GDNF IgG treatments down-regulate TRPV1 expression in nociceptors and reduce thermal hyperalgesia (Fang et al., 2003; Amaya et al., 2004). Deletion of GFRα2 in mice also reduces inflammatory pain (Lindfors et al., 2006). Recent studies have shown GDNF, NTN and ART can potentiate TRPV1 function as well as induce capsaicin responses in a subset of DRG neurons that are normally capsaicin-insensitive, and injection of GDNF, NTN or ART can all produce acute thermal hyperalgesia in mice (Malin et al., 2006). These studies suggest that GDNF family growth factors can modulate nociceptor responses. Indeed, TRPV1 is expressed in 99% of GFRα3-positive, artemin responsive neurons in mice. In addition, overexpression of ART in the skin of transgenic mice not only increased gene expression of TRPV1 in sensory ganglia but also increased the density of TRPV1-positive afferents in the skin (Orozco et al., 2001; Elitt et al., 2006). An increased response of DRG neurons to capsaicin, a decreased thermal threshold of cutaneous nociceptors and an increased behavioral response to thermal stimuli has also been measured in mice that chronically overexpress artemin (ART-OE) (Elitt et al., 2006). These findings suggest a critical role of ART in regulation of TRPV1-mediated nociception

GFLs have also been found to be effective in reversing some of the changes in afferent phenotype and hypersensitivity that accompany nerve injury. In these neuropathic pain models, constriction of a peripheral nerve produces a pathological condition that leads to a persistent pain state. Nerve constriction injury is known to increase expression of the GDNF receptor GFRα1 and the ART receptor GFRα3 in the damaged DRG (Bennett et al., 2000). This increase is thought to be due to the interruption of the normal retrograde trophic signaling from the target that is blocked by the constricted nerve. Intrathecal injection of GDNF or subcutaneous injection of ART reduces injury-related expression of pain-associated sensory neuron markers and relieves much of the behavioral hypersensitivity associated with this injury. This relief is hypothesized to occur through activation of signaling pathways that act to reduce sodium channel activity in injured DRG neurons and through inhibition of neurotransmitter release in the dorsal horn (Boucher et al., 2000; Gardell et al., 2003). Thus, restoration of pre-ligation trophic signaling,

which is interrupted by the constriction, restores normal sensitivity and produces an analgesic effect. These studies provide evidence for functional down-regulation of nociceptor properties by GDNF family factors such as ART, whose role in pain signaling in the aging system is a major focus of this dissertation.

II. INTRODUCTION
AGING IN THE PERIPHERAL NOCICEPTIVE SYSTEM

With the population of elderly people rapidly increasing in society, studies of aging and associated changes in the nervous system have become more essential. Neurological diseases are common in the aging population and account for about 50% of the disability reported in the elderly (Verdu et al., 2000). Aging has been defined as a process of "accumulation of diverse deleterious changes in the cells and tissues with advancing age that increase the risk of disease and death"(Harman, 2001). Based on this definition aging affects all tissues and cells, and causes tissue damage, cell loss and function reduction. For the nervous system aging results in decline of sensory, motor and cognitive functions with time, indicating all regions, including the CNS and the PNS, are affected. However, cells in the nervous system are differentially affected during aging, and neurons in some regions are more vulnerable than others, a phenomenon called selective neuronal vulnerability (Mattson and Magnus, 2006).

Although the mechanisms for this selective neuronal vulnerability remain unknown, it suggests that aging mechanisms in different regions and/or different types of neurons may vary, and this may also be true in the PNS. Some studies have shown that age-related changes in the structure and function of the peripheral sensory system affect pain sensation. However, the effect of age on pain remains elusive and controversial. Moreover, the mechanisms that underlie aging in the PNS are much less understood since most aging studies focus on the CNS. To understand the molecular mechanisms underlying aging in the PNS, this dissertation examined age-related changes at the molecular and cellular level using a mouse model system. Results indicate that changes in artemin/GFRα3 expression and the baseline level of inflammatory protein expression may contribute to age-related deficits in the peripheral nociceptive system.

2.1 AGE-RELATED CHANGES IN THE PERIPHERAL SENSORY SYSTEM

2.1.1 Behavioral changes

Many studies have been done on cutaneous sensitivity for noxious thermal, mechanical and electrical stimuli with age in normal human and animal models. Although some have reported either no age-related differences in pain sensitivity or decreased pain thresholds and increased sensitivity with age (Kenshalo, 1986; Heft et al., 1996; Jourdan et al., 2000; Iwata et al., 2002; Kitagawa et al., 2005), most studies have shown that older subjects are less sensitive to noxious stimuli than younger subjects (Gibson and Farrell, 2004). Studies also have investigated the effects of age on pain sensation under pathological conditions such as inflammation and nerve injury (Gagliese and Melzack, 2000). In some studies, aged animals had greater thermal hyperalgesia and/or mechanical allodynia compared to younger ones (Crisp et al., 2003; Zhang et al., 2004) while other studies showed similar or decreased responses to tissue injury in aged animals compared with young animals (Kitagawa et al., 2005). The lack of consensus in the effect of aging on pain sensitivity may be due to methodological differences (Helme et al., 2004), including modality, duration and sites of stimulation, models of inflammation and nerve injury, and the time points of behavioral tests. However, clinical studies indicate that pain is more frequently absent in older patients with myocardial infarction, pneumothorax, and peptic ulcer diseases (Gibson and Helme, 2001; Moore and Clinch, 2004). Nonetheless the weight of all evidence supports the conclusion that pain thresholds increase and sensitivity to noxious stimuli decreases with age. Pain is a key pointer to disease diagnosis in the clinic, and decrease or even absence of pain in some diseases may have critical implications. Though multiple interacting neurobiological and behavioral factors contribute to the effects of aging on pain sensitivity, the age-related functional deficits in pain sensation may be a consequence of age-induced degeneration in the peripheral nociceptive system, since both human and animal studies have reported that the peripheral sensory system undergoes age-related degenerative changes in both structure and function.

2.1.2 Anatomical and structural changes

Age-related anatomical and structural changes in the PNS include loss of neurons and axons. One study found about 34% loss of total lumbar DRG neurons and 57% loss of DRG neurons innervating the knee joints in 24-month old mice (Salo and Tatton, 1993). However, most studies demonstrated that there was no decrease or only a small non-significant decrease (~12%) of total primary sensory neurons in DRG of 22~30-month old rats (La Forte et al., 1991; Bergman and Ulfhake, 1998; Mohammed and Santer, 2001). Thus, age-related sensory deficits are not simply of a result of neuronal death. Morphologic studies of peripheral nerves show a reduction in the number and density of myelinated as well as unmyelinated fibers of several animal species with aging (Bergman and Ulfhake, 2002; Besne et al., 2002; Ulfhak et al., 2002; Vilches et al., 2002). In human, both myelinated and unmyelinated fibers have been reported to decrease, with loss of unmyelinated fibers greater (~50%) compared to myelinated afferents (~35%) in very old age (65-75 years) (Verdu et al., 2000). This decrease appears to be site specific, and the degree of loss is greatest in the distal regions of long nerves (Flanigan et al., 1998). In parallel with this loss of nerve fibers in peripheral nerves, a significantly lower density of epidermal nerve profiles has been reported in adult and aged healthy human subjects than in young humans (McArthur et al., 1998). A decrease of 50% of nerve profiles was also reported in plantar skin of 24-month old rats (Verdu et al., 2000). In contrast, only a moderate reduction of about 10~15% in the density of unmyelinated sensory fibers was found in the epidermis of 18-month old mice with respect to young mice (Verdu et al., 2000). This may be due to compensation for the loss of terminal innervations by sprouting and expansion of the target territory, a compensatory mechanism particularly effective for thin nerve fibers. Similarly, myelinated and unmyelinated input to the spinal cord also decreases in aged rats, and the loss of myelinated fibers is greater than unmyelinated (Bergman and Ulfhake, 2002).

2.1.3 Electrophysiological changes

Physiological changes have been reported in peripheral sensory nerves of aged human subjects. Electrophysiological measures show slower nerve conduction velocity and smaller nerve action potential amplitude in older subjects compared to young individuals, and this decrease begins in

early adulthood and progresses in a relatively stable pattern (Flanigan et al., 1998; Verdu et al., 2000). Although it has been shown that both C-fiber and A-fiber function decrease with age, one study found that selective block of A-fiber conduction while leaving C-fiber function intact increased heat pain threshold in young adults without effects on older subjects, suggesting a selective age-related impairment of myelinated nociceptive A-fiber function (Chakour et al., 1996). Studies of calcium currents in rat DRG neurons found reduction of calcium current and an increase in the percentage of high-threshold calcium currents in 30-month old rats (Kostyuk et al., 1993). Consistent with these findings, a depolarization–induced increase in intracellular calcium transients in old neurons was also lower compared with cells isolated from 7-month-old rats (Kirischuk et al., 1992). Effects of aging on electrical membrane properties (EMP) of cultured mouse DRG neurons have been well studied (Scott et al., 1988). Old neurons have a number of significant alterations in EMP compared with young ones, including decreased electrical excitability and increased action potential duration. The pattern of altered EMP is consistent with an age-induced shift from voltage-sensitive sodium channels to less excitable voltage-dependent calcium channels (Scott et al., 1988). These findings strongly suggest that age-induced changes in neuronal sensitivity and excitability may result from altered expression and/or function of ion channels with aging.

2.1.4 Cellular and molecular changes

Few studies have investigated age-related cellular and molecular changes in the PNS. In lumbar and cervical DRG of aged rats there were decreased cellular levels of CGRP and substance P, two major neurotransmitters of primary afferent nociceptive fibers (Bergman et al., 1996). The rate of CGRP axonal transport also decreased with aging (Fernandez and Hodges-Savola, 1994; Hukkanen et al., 2002). Neurotrophic support is important for maintaining functional properties of the adult nervous system, and changes of neurotrophic signaling in the aged PNS have been reported. A decrease in mRNA and protein level of TrkA, TrkB and TrkC was observed in DRG of aged rats, and this decrease was more remarkable in lumbar DRG (Bergman et al., 1996; Bergman et al., 1999a). Consistent with these findings, axotomy results in down-regulation of these neurotrophin receptors and the effect of axotomy is less pronounced in aged animals than in young adults (Bergman et al., 1999a). One study found up-regulation of the GDNF receptor

GFRα1 and RET in primary sensory neurons (Bergman et al., 1999b). These observations indicate that alterations of neurotrophic signaling may be involved in age-related neuronal changes.

In summary, there are significant age-related changes in the peripheral nociceptive system, indicated by decreased number and conduction velocity of sensory nerve fibers, decreased neuronal excitability and decreased neurotransmitter production. All these changes reflect a reduction in function and likely contribute to decreased pain sensitivity. Although some studies found loss of neurotrophic support, others have not. Thus, a lack of neurotrophic signaling may not totally account for the age-induced decrease in function of the PNS.

2.2 POSTULATED MECHANISMS OF NEURONAL AGING

Multiple factors, including developmental/genetic and environmental factors, can contribute to age-related changes in cellular function. The aging process is postulated to represent an inherent complex process manifested at genetic, molecular, cellular, organ and system levels within an organism. In the nervous system the aging is reflected by a decrease in neuronal sensitivity to stimulation. To date the fundamental mechanisms of the inherent aging process are still poorly understood. Many mechanisms have been proposed for neuronal aging, including changes in oxidative stress, calcium homeostasis, inflammation, and the neuroendocrine system, although none has been completely successful in explaining the aging process of neurons.

2.2.1 Oxidative stress – mitochondria theory

Harman first proposed in the 1950s that damage to cellular macromolecules by free radicals produced in aerobic organisms is a major determinant of lifespan. The discovery of the contribution of reactive oxygen species (ROS), some of which are not free radicals, to oxidative damage to cellular constituents including lipids, nucleic acids and proteins, led to a modern

version of this tenet – the oxidative stress theory (Kregel and Zhang, 2006). The primary intracellular sites for ROS production *in vivo* are mitochondria. The mitochondria theory proposes that mitochondria are the critical component in control of aging. It hypothesizes that ROS can damage mitochondrial DNA (mtDNA) and components, leading to further increase in intracellular ROS levels and a decline in mitochondrial function. This causes cellular energy deficits and impaired normal cellular activity and compromises the ability of the cell to adapt to various physiological stresses (Shigenaga et al., 1994). The mitochondria theory has been supported by many studies of age-related changes in mitochondria in multiple organs of different species (Shigenaga et al., 1994; Kregel and Zhang, 2006). The most common and consistent findings show increased mtDNA damage with aging (Shigenaga et al., 1994; Hamilton et al., 2001). The mitochondrial genome contains 37 genes and 13 of them encode proteins involved in the electron transport chain (Chan, 2006). Thus, accumulation of mtDNA defects can account for the age-related deficits in mitochondrial bioenergetic capacity and function.

Neurons appear particularly vulnerable to mitochondrial dysfunction (Chan, 2006). Neurons are enriched in unsaturated lipids, which are prone to oxidative damage. Accumulation of lipid damage can decrease fluidity and increase rigidity of plasma membranes, and lead to a decline in membrane receptor-mediated signaling and in turn a decrease in function (Shigenaga et al., 1994). Moreover, age-related mtDNA deletions have been found in both the CNS and the PNS (Blanchard et al., 1993; Nickander et al., 2002). Recently two studies reported high levels of mtDNA deletions in aged human substantia nigra neurons, suggesting that mtDNA damage can selectively contribute to neuronal aging (Bender et al., 2006; Kraytsberg et al., 2006). A study of the PNS also showed that the levels of a particular deletion in mtDNA in DRG was about 300-fold higher in 24month-old rats compared to young rats, and the abundance of this deletion strongly correlated with age-related decline in sensory function (Nagley et al., 2001). Besides mtDNA, oxidative stress also affects nuclear DNA in the nervous system. Some genes, such as calmodulin 1 and sortilin, are selectively vulnerable to oxidative damage in human neurons, and expression of these genes was decreased in human frontal cortex after age 40. These genes play central roles in synaptic plasticity and vesicular transport, and reduced expression may be directly involved in functional deficits of the aged brain (Lu et al., 2004).

2.2.2 Inflammation theory

The fact that the aging process is accompanied by an increased incidence of various chronic diseases leads to a supplemental theory to oxidative stress – the molecular inflammation theory of aging, which connects biological aging with age-related pathological conditions. Inflammation represents a complex defense of an organism to intrinsic and extrinsic insults and stress. Accumulation of oxidative damage appears to be a primary causal factor in producing a state of chronic inflammation during aging (Lavrovsky et al., 2000; Chung et al., 2006; Sarkar and Fisher, 2006). At the molecular level the primary markers for chronic inflammation are high levels of inflammatory mediators, such as, IL-1, IL-6 and TNFα. Activation of transcriptional factors like NF-κB by age-related oxidative stress causes up-regulation of inflammatory gene expression and inflammatory molecule production, results in inflammation processes and inflammation-induced cellular and tissue damage, and contributes to the pathogenesis of age-related diseases (Chung et al., 2001; Chung et al., 2006). There is substantial evidence supporting the link between aging and chronic inflammation in a wide range of tissues, organs, systems and species including humans. It has been well documented that inflammatory molecules are increased with advancing aging, though the level of inflammatory markers in age-related chronic inflammation is much lower than the levels generated during acute inflammatory conditions (Kregel and Zhang, 2006).

Association of inflammation to neuronal aging in the CNS has been well studied. Increased expression of inflammatory genes with aging has been detected in various regions of the brain (Lee et al., 2000; Erraji-Benchekroun et al., 2005; Frank et al., 2006). In the CNS glial cells including astrocytes and microglia play an important role in inflammation processes. Activated glial cells produce multiple inflammatory cytokines like IL-6, IL-1 and TNFα, which are deleterious to neurons (Krabbe et al., 2004). Epidemiological studies also have shown association between high levels of inflammatory cytokines such as IL-6 and poor cognitive functions in aged subjects (Yaffe et al., 2003). Long-term use of anti-inflammatory drugs decreases age-related increases in inflammatory markers and prevents cognitive decline (Casolini et al., 2002; Etminan et al., 2003; Yaffe et al., 2003). Caloric restriction or diets enriched in

antioxidants not only reduce oxidative damage but also dramatically decrease inflammatory responses and reverse age-induced decline in neuronal function, supporting the roles of inflammation in the aging process of neurons (Lee et al., 2000; Gemma et al., 2002).

2.2.3 Calcium theory

Besides energy production, mitochondria also control intracellular calcium levels. Age-induced mitochondrial dysfunction can cause intracellular calcium dysregulation, which may be involved in neuronal aging. The calcium hypothesis of neuronal aging arose from awareness of the neurotoxic effects of elevated calcium (Schanne et al., 1979). Now it is well known that the free intracellular Ca^{2+} concentration plays a major role in neuronal signal transduction. Elevation of intracellular Ca^{2+} activates presynaptic neurotransmitter release, regulates membrane excitability, and modulates the activity of various second messenger systems and gene expression (Hartmann et al., 1996). The calcium theory postulates that age-dependent dysregulation of calcium homeostasis that result in changes in the free intracellular Ca^{2+} concentration account for the age-related changes in neuronal functions (Biessels and Gispen, 1996). Regulation of intracellular calcium involves a complex and integrated set of systems including plasma membrane (calcium channels and transporters), intracellular calcium buffering (calcium binding proteins) and intracellular storage sites (mitochondria and endoplasmic reticulum ER). It is well recognized that multiple factors, such as increased calcium influx and impaired mitochondrial and ER function, contribute to age-related calcium dysregulation in neurons (Thibault et al., 1998).

The relationship between calcium influx via membrane calcium channels and neuronal function in aging has been well studied in the CNS. Most studies support the idea that age-dependent alterations of calcium channels result in changes in intracellular Ca^{2+} concentration and account for the age-related changes in neuronal function like memory and learning (Griffith et al., 2000; Toescu and Verkhratsky, 2003, 2004; Toescu et al., 2004). The consistent and predominant findings in aged neurons include delayed recovery of intracellular Ca^{2+} and increased amplitude/duration of afterhyperpolarization (AHP) following stimulation (Disterhoft et al., 1996; Kirischuk and Verkhratsky, 1996). AHP is mediated by activation of Ca-dependent

K currents and has an inhibitory effect on neuronal excitability. The delayed recovery and increased intracellular Ca^{2+} at the end of stimulation extend the period of Ca-dependent K channels activation and result in a large and prolonged AHP, decreasing excitability of aged neurons. Further studies provided strong evidence that the changes in AHP with aging were due to increased high threshold VGCCs rather than K channels (Campbell et al., 1996; Landfield, 1996; Thibault and Landfield, 1996). Similar changes with slow recovery of intracellular Ca^{2+}, increased AHP and decreased electrical excitability also have been found in DRG with aging (Scott et al., 1988; Kirischuk and Verkhratsky, 1996). Interestingly, VGCC-mediated calcium influx is increased in aged neurons of mammals (Murchison and Griffith, 1995; Thibault and Landfield, 1996), and some anticonvulsants, which act on certain neuronal calcium channels, have been discovered to extend worm life span and retard the aging process, implicating that VGCC-mediated neural activity may be involved in aging (Evason et al., 2005).

2.2.4 Neurotrophic theory

Thus far none of the three theories discussed above can explain very well a common observation in the aging process of the nervous system - the selective neuronal vulnerability. In the CNS subcortical regions exhibit greater vulnerability to aging than cortical regions (Smith et al., 1999). In the PNS loss of unmyelinated fibers during aging is greater than loss of myelinated fibers (Verdu et al., 2000). In the autonomic nervous system even relatively homogenous populations of neurons show diverse changes in old age (Cowen, 2002). The neurotrophic theory proposes that age-induced changes in neurotrophic factor signaling pathways may contribute to age-related selective neuronal vulnerability (Cowen, 1993; Gavazzi and Cowen, 1996). As mentioned before, neurotrophic factors are essential for the development and maintenance of discrete population of neurons in the PNS and CNS. Both peripheral targets and cells in the nervous system produce neurotrophic factors and promote neuronal survival and synaptic plasticity. Either decreased neurotrophic factor production or altered signal transduction may have a role in neuronal aging. Although this theory explains the selective neuronal aging to some degree, it remains controversial despite some supporting evidence.

Age-related decreases in the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus have been reported (Gooney et al., 2004; Hattiangady et al., 2005). However, other studies did not find any age-related changes in gene expression of either BDNF and NGF or their receptors in the hippocampus (Lapchak et al., 1993; Rylett and Williams, 1994). The more consistent finding is the reduced responses of aged brain to neurotrophic factors (Smith, 1996; Mattson and Magnus, 2006). For example, up-regulation of neurotrophic signaling in response to injury is impaired in aged animals (Scott et al., 1994; Smith and Cizza, 1996; Yurek and Fletcher-Turner, 2000). Age-related changes in neurotrophic factor receptors also have been reported in the PNS (see **section 2.1.4**). Further studies showed decreased levels of neurotrophin including NGF and BDNF mRNAs in target tissues of aged rats, consistent with previous findings of age-related impairment in neurotrophic signaling, but GDNF was found strongly up-regulated in target tissues (Ming et al., 1999b, 1999a). Moreover, in vitro studies indicated that responses of aged DRG neurons to NGF treatment were similar to young neurons, suggesting that NGF-mediated neurotrophic signaling was not damaged in aged sensory neurons (Jiang and Smith, 1995; Jiang et al., 1995; Hall et al., 2001). Therefore whether there is loss of neurotrophic support in the aged nervous system remains unclear.

2.3 HYPOTHESES

The postulated mechanisms for neuronal aging provide clues to understand the aging process of the peripheral nociceptive system. These theories (mitochondrial, calcium, neurotrophic support) interrelate with each other and likely have overlapping mechanisms that contribute to the aging process (Macdonald et al., 2000). In the PNS, prior studies have suggested that loss of neurotrophic support has a major role in aging of the PNS. Although we began this study with this hypothesis in mind, evidence from studies done in recent years indicates that neurotrophic factors in the adult system have roles other than ones of survival, i.e., they may also affect neuron responsiveness. Thus, although GDNF-family ligands (GFLs) support the survival and differentiation of specific subtypes of developing sensory neurons, in the adult PNS, the role of GFLs may change to one in which they modulate sensory neuron response properties,

particularly following inflammatory or neuropathic injury to the system (see **section 1.3.2**). As mentioned, increased GFRα1 has been reported in lumbar DRG neurons of naïve aged rats, supporting a role whereby elevated GFRα1 signaling down-regulates nociceptor function and decreases afferent sensitivity, in line with its putative role in reducing neuropathic pain in the young PNS. Similarly, the effects of ART/GFRα3 signaling on nociceptor function during aging might also influence the loss of behavioral sensitivity in aged mice. With this in mind, we investigated the role of GFLs in the aging sensory system to determine whether they may contribute to the reduced behavioral responses exhibited by older animals.

The physiological function of nociceptors depends on their distinct expression of channel proteins such as TRPV1 and Nav1.8. In mouse DRG TRPV1 is mainly expressed in NGF-dependent nociceptors and 67% of TRPV1-postive neurons also coexpress the ART receptor GFRα3, whose expression is restricted to the PNS (Yang et al., 2006). Thus, NGF and ART likely have major roles in the regulation of TRPV1 expression and function in the PNS of mice. Since no major loss of NGF-dependent neurotrophic expression occurs in the aged PNS and GDNF signaling does not affect nociceptors expressing TRPV1 in mice (Zwick et al., 2002), we hypothesized and tested whether altered ART/GFRα3 expression in the PNS during aging correlated with the level of expression and/or function of ion channels associated with nociception, such as TRPV1, in cutaneous nociceptors.

Results to be presented in this study also indicate that age predominantly up-regulates inflammation and immune-related mRNAs in the trigeminal sensory ganglia, suggesting that inflammation has a major role in aging of primary sensory neurons. In the nervous system, infiltrating immune cells and glial cells produce inflammatory cytokines and contribute to inflammation. These cells also produce growth factors such as NGF, which is a known inflammatory mediator, suggesting that neurotrophic factors derived from immune or immune-like cells in the sensory ganglia may be involved in age-related inflammatory changes. We therefore investigated whether expression of immune-related genes was altered in the aged sensory system.

To test these hypotheses, we first investigated whether expression of ion channel and receptor proteins associated with nociception and neurotrophic signaling were modified in normal aged animals (**Chapter IV**). We then examined whether age-related modification of neurotrophic signaling and channels and receptors affected inflammation-induced pathological pain (**Chapter V**). These studies were followed by an investigation into whether the neurotrophic factors ART and NGF affected the functional properties of nociceptive channels such as TRPV1 in sensory neurons of aging animals (**Chapter VI**). Our results indicate that altered ART/GFRα3 signaling in the PNS leads to down-regulation of TRPV1 expression and function in nociceptors, which in turn leads to a reduction in thermal sensitivity and inflammation-induced thermal hyperalgesia. These findings suggest a critical role of ART/GFRα3 signaling in modulation of TRPV1-dependent thermal sensation in the aging PNS.

III. MATERIALS AND METHODS

3.1 ANIMALS AND BEHAVIORAL TESTING

3.1.1 Animals

Young (6~8week), middle-aged (15~18month) and aged (2year) male and female C57BL/6NIA (B6) mice were obtained from the aging rodent colony supported by the National Institute on Aging at Harlan (Indianapolis, IN, USA). Upon arrival at the University of Pittsburgh animal facility, mice were group housed in microisolator caging and maintained on a 12-h light/dark cycle in a temperature-controlled environment (20.5 °C) with access to food and water ad libitum. These studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the NIH Guide for the Care and Use of Laboratory Animals.

3.1.2 Complete Freund's adjuvant-induced inflammation

Detailed methods for this procedure have been reported previously (Zwick et al., 2003). Briefly an emulsion of complete Freund's adjuvant (CFA) was prepared by thoroughly mixing equal volumes of CFA (heat killed and dried *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate (Sigma, St Louis, MO) with sterile saline. Twelve 6 week-old and twelve 16 month-old mice lightly anesthetized with isoflurane received a subcutaneous injection of the CFA emulsion (~20 μl) in the plantar surface of the hind-paw. To determine the extent of edema, the diameter of the hind-paw was measured using a caliper square prior to injection and each day after behavioral testing. All mice showed substantial edema after CFA injection.

3.1.3 Hargreaves' test of thermal sensitivity

Mice were placed in individual chambers (10.0 cm long × 10.0 cm wide × 13.0 cm high) of a 16-chamber plexiglas container that was placed on top of a 6.0 mm thick glass surface (Model 390; IITC Inc., Woodland Hills, CA) maintained at 30°C and allowed to acclimate for 1–2 h before testing. Response latencies to noxious thermal stimulation were measured by applying a radiant heat stimulus (setting at 20% intensity for normal behavior test; for CFA study setting was at 12% for young animals and 15% for old animals) to each hind-paw. Different heat settings were used in the CFA study to insure responses after CFA injection could be measured in an accurate manner, i.e., they were greater than 3 s. The heat source was activated with an electric trigger coupled to a timer, and the latency to stimulus response (flinching or lifting the paw) was recorded to the nearest 0.1 s. Mice were tested twice, and the responses for each paw were averaged. The left and right hind paw was tested on each mouse once a day for three consecutive days. CFA-injected animals were tested once a day, 3 days prior to CFA injection, then every other day for 1 week (days 1, 2, 3, 5, 7). After behavioral testing animals were given an overdose of Avertin (2-2-2 tribromoethanol in tert-amyl alcohol) anesthetic and perfused transcardially with 75 ml of ice-cold 0.9% saline. Hind-paw skin, nerves (tibial, sciatic or saphenous), DRG (either pooled from all levels or pooled from lumbar levels L3/L4/L5) and/or TG were collected on dry ice for RNA/protein analysis.

3.2 GENE EXPRESSION ANALYSIS

3.2.1 RNA isolation

RNA was isolated by homogenizing frozen tissue in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation. Pellets were washed with 70% ethanol, suspended in RNase-free water and in some cases run on an RNeasy column (Qiagen). The concentration was determined using a GeneQuant RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ). RNA (5 µg) was treated with DNase (Invitrogen) to remove genomic DNA, and then 1 µg was reverse-transcribed using Superscript II reverse transcriptase (RT) (Invitrogen).

3.2.2 Microarray Assay

Detailed methods for this procedure have been reported previously (Lee et al., 1999). Briefly ten micrograms of RNA were synthesized into cDNA and then in vitro transcribed into biotin-labeled cRNA. Twenty micrograms of cRNA were collected and brought to the University of Pittsburgh Macromolecular Analysis Faculty, and hybridized to Affymetrix mouse gene chips using protocols suggested by the manufacture. Affymetrix mouse genome U74Av2 oligonucleotide microarrays representing 12,423 known transcripts and expressed sequences (ESTs) were used. Data were analyzed using dChip software (Harvard University). Fold differences between the mean signals were calculated as max/min with down regulation relative to young groups expressed as negative. Significance was set at $P < 0.05$

3.2.3 Radioactive RT-PCR analysis

RT-PCR reactions were done in the presence of ^{32}P-dCTP and aliquots of the reaction run on 8% polyacrylamide gels in Tris borate EDTA buffer. Gels were dried and placed against a PhosphorImager screen, and the relative level of incorporated label was determined using a Bio-Rad PhosphorImager (Hercules, CA). The cycle number was optimized for each set of primers by first running PCR reactions at different cycle numbers to establish the midphase of the reaction. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sets were generated using Macvector software (Accelrys, San Diego, CA). Routine control reactions included PCR reactions on DNased RNA (without RT) and reactions run without templates to test for contamination. Primer sequences included the following (5' and 3', respectively): NGF, tccaatcctgttgagagtgg and caggctgtgtctatgcggat; ART, ctcagtctcctcagcccg and tccacggtcctccaggtg; GDNF, aaggtcaccagataaacaagcgg and tcacaggagccgctgcaatatc; and GAPDH, atgtgtccgtcgtggatctga and gctgttgaagtcgcaggagaca.

3.2.4 Real-time PCR

SYBR Green labeled PCR amplification was performed using a real-time thermal cycler (Applied Biosystems, Foster City, CA) controlled by a Dell Latitude laptop computer running

Table 3.1 Primer sequences used for real-time PCR assays

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
GFRα1	GCCACTCCTGGATTTGCTGATG	AAGTTGGTTTCCTTGCCCGC
GFRα2	GGAAAGCCCATTGTATGACTGCC	TGAAGCGAGCCTGAAGATGTCC
GFRα3	CTTGGTGACTACGAGTTGGATGTC	AGATTCATTTTCCAGGGTTTGC
RET	ACTCGGCTCTCTGAGATAGACA	AGACCTTGGTCCAGGTCAACAA
TrkA	AGAGTGGCCTCCGCTTTGT	CGCATTGGAGGACAGATTCA
TRPV1	TTCCTGCAGAAGAGCAAGAAGC	CCCATTGTGCAGATTGAGCAT
TRPV2	CCAGCCATTCCCTCATCAAAA	AAGTACCACAGCTGGCCCAGTA
TRPV3	TGAAAGAAGGCATTGCCATTT	GAAACCAGGCATCTGACAGGAT
TRPV4	TGGATTCCTTGTTCGACTACGG	CACAATGTCAAAGAGGATGGGC
Nav1.8	GCCACCCAGTTCATTGCCTTTTC	TCCCCAGATTCTCCCAAGACATTC
Nav1.9	TCTTCCTGGGCTCTTTCTACCTGC	CATCTTCTCCTTGGCTTCTGTCTCG
Cav2.2	ATCCGCATCCTATTGTGGACC	GTCATCATCAAGGGCACTGTTTC
Cav2.2-e37a	TACCTCACTCGGGACTCTTCCATC	CGCAATACAACGCAACAAACTG
P2X3	TCCTACTTTGTGGGGTGGGTTTTC	TCTGTTGGCATAGCGTCCGAAG
ASIC3	TTCGCTACTATGGGGAGTTCCAC	GCAGGGGATTGATGTTACACAAAG
IL-6	TCAATTCCAGAAACCGCTATGA	CACCAGCATCAGTCCCAAGA
LIF	AGAATCAACTGGCACAGCTCAATGG	ACATAGCTTTTCCACGTTGTTGGG
ETRB	ACCCTGATGACCTGCGAAATGC	ACAGAGAGCAAACACGAGGACCAG
Myelin	CTTCAATACCTGGACCACCTGTCAG	GTCATTTGGAACTCGGCTGTTTTG
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT

ABI Prism 7000 SDS software. Twenty nanograms of cDNA template were added to 50μl reaction mixtures provided in the SYBR Green reagent kit (Applied Biosystems). The amplification protocol included 2 min at 50 °C to activate the AmpErase UNG to prevent the reamplification of any carryover PCR products, 12 min at 95 °C to activate the Amplitaq polymerase, 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. After amplification, a dissociation curve was plotted against melting temperature to ensure amplification of a single product and to test for primer dimers. All samples were run in duplicate. Controls were run with water replacing the template (to further test for primer dimers). The CT values for each reaction were obtained and the ΔCT was calculated by subtraction of

control (GAPDH) CT from the experimental value. $\varDelta\varDelta CT = $ Mean $\varDelta CT$ young $-$ Mean $\varDelta CT$ old and fold change $= 2{-}\varDelta\varDelta CT$. An unpaired t-test ($P \leq 0.05$) was used to determine significance of expression. PCR primers were generated using Primer Express software (Applied Biosystems, Foster City, CA) using parameters optimized by the manufacturer.

3.3 PROTEIN ANALYSIS

3.3.1 Western immunoblotting

Isolated nerves and DRG (either pooled from all levels or pooled from lumbar levels L3/L4/L5) were analyzed. Tissues from young and aged animals were homogenized in lysis buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM Tris–HCl (pH 7.4), 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM sodium orthovanadate and 100 µg/ml phenylmethylsulfonyl fluoride (Sigma Biochemicals). Homogenates were spun 10 min at 10,000 rpm at 4 °C, the supernatant recovered and protein concentration determined using a Bio-Rad protein assay. Samples (10–20 µg of protein) were boiled 10 min in denaturing buffer containing β-mercaptoethanol and SDS, separated on either 7.5 or 10% polyacrylamide SDS-page gels and transferred to Hybond-P PVDF membrane (Amersham Life Sciences) that was blocked for 1 h in TBS solution containing 5% powdered milk, 0.01% Tween-20, pH 7.6. Membranes were incubated with primary antibodies overnight at 4 °C. Antibodies used were: rabbit anti-TRPV1 (Oncogene Research Products; 1:500), goat anti-GFRα3 (R&D system, 1:500), rabbit anti-β actin (Abcam, 1:16000), rat anti-tubulin (sera-lab, 1:100) and rabbit anti-Nav1.8 (1:1000, a gift from Dr. S. Waxman, Yale University). Antibody binding was visualized using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) and ECL plus detection (Amersham Life Sciences). Immunoreactive bands were analyzed by densitometry and their intensity quantified using NIH Image J software. Immunoblot band intensity was normalized to either tubulin or actin or protein bands from each sample visualized on Coomassie blue stained gels. Bands at the approximate molecular weight of the protein of interest were chosen for comparative measure.

3.3.2 Immunocytochemistry

Tissues (DRG, TG, nerve and skin) were removed from young, aged and TRPV1 knockout animals that were perfused with saline. Samples were placed in 25% sucrose made in 0.1 M phosphate buffer (PB) overnight at 4 °C and then embedded in Optimal Cutting Temperature (OCT; Tissue Tek) compound on dry ice. Twenty-micron cryostat sections were mounted on Superfrost/Plus slides (Fisher), fixed in 4% paraformaldehyde for 10 min, blocked in 5% NGS, 2% BSA and 0.25% Triton X-100 for 1 h and then incubated in primary antibody (rabbit anti-TRPV1, 1:250, Oncogene Research; goat anti-artemin, 1:60, R&D system; goat anti-GFRα3, 1:200, R&D system, rabbit anti-ETRB, 1:500, Abcam) overnight at room temperature. Antibody binding was visualized by avidin–biotin–peroxidase complex formation or using Cy2- or Cy3-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). The percentage of TRPV1-positive or GFRα3-positive neurons was determined by first capturing entire, nonconsecutive labeled sections of the L4/L5 ganglia using a camera mounted on a microscope and Photoshop software. NIH Image software was then used to circle both labeled and unlabeled neurons to determine size and density of immunolabeled cells.

3.4 CALCIUM IMAGING

3.4.1 Cell culture

Detailed methods for this procedure have been described in previous studies (Malin et al., 2006). 2~3-month old (young) and 16~18-month old (middle-aged) male Blk6 mice from the NIA colony were used. All levels of DRG were dissected and placed in cold Ca^{2+}/Mg^{2+}-free HBSS. After consecutive incubation in 20U/ml papain solution and 4mg/ml collagenase solution for 10 min at 37°C, DRG were triturated and dissociated in serum-containing media with fire-polished glass pipettes, and then plated onto laminin/poly-lysine coated glass coverslips. Dissociated cells on the coverslips were incubated at 37°C for 2 h to allow cells to attach to the coated surface. Cells were fed with growth media containing either 50ng/ml NGF (Harlan BioProducts) or

50ng/ml NGF plus 250ng/ml artemin (Biogen IDEC). DRG cultures were incubated for less than 24 h in a CO_2 incubator at 37°C before used in the calcium imaging experiments.

3.4.2 Calcium imaging

Isolated sensory neurons were loaded with 2 μM fura-2 in HBSS containing 5 mg/ml BSA for 30 min at 37°C and then mounted on an Olympus Optical (Thornwood, NY) upright microscope stage with constantly flowing buffer at 5 ml/min. Perfusion rate was controlled with a gravity flow system (VC66; Warner Instruments, Hamden, CT), and perfusate temperature was maintained at 30°C using a heated stage and an in-line heating system (PH1, SHM-6, TC344B; Warner Instruments). Drugs were delivered with a rapid-switching local perfusion system. Firmly attached, refractile cells were identified as regions of interest in the software (Simple PCI, C-Imaging; Compix Imaging Systems, Sewickley, PA). All fields were first tested with brief application of 50 mM KCl, and Ca^{2+} transients were imaged to standardize pipette placement and to ensure that cells were healthy and responsive. Responses were measured as the ratio of absorbance at 340 nm to that obtained at 380 nm ($\Delta F_{340/380}$) [DG4 (Sutter Instruments, Novato, CA); Retiga 1300 (Burnaby, British Columbia, Canada)]; peak responses were >0.2 $\Delta F_{340/380}$ and were easily distinguished from optical noise (< 0.02 $\Delta F_{340/380}$).

3.4.3 Protocols

Application of 50mM KCl causes an increase in intracellular Ca^{2+} and was used to distinguish neurons from non-neuronal cells and to insure neuron viability. 50mM KCl was applied for 5s at the beginning of all experiments and only cells responsive to KCl application were analyzed. To test if CAP has direct effects on internal Ca^{2+} release, 1uM CAP was applied for 5s to cells in Ca^{2+}-free HBSS (**Figure 3.1A**). For most experiments CAP was applied for 5s three times every 10 min. To test potentiation by ATP, 100uM ATP was applied for 30s at 7 min after the 2nd CAP application (**Figure 3.1B**). To test potentiation by ART, a 7-min perfusion of 100ng/ml ART was applied before the 2nd application of CAP (**Figure 3.1C**).
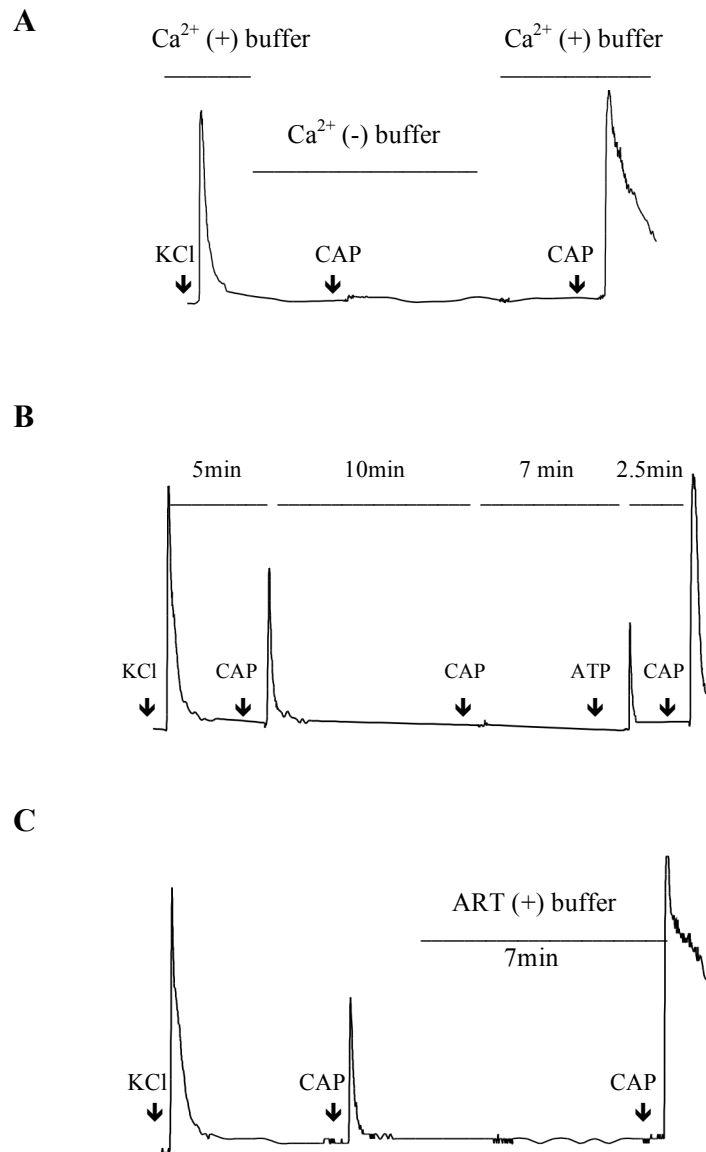
Figure 3.1 Protocols used in Ca^{2+} imaging experiments. **A.** Extracellular Ca^{2+}-dependent CAP response: 1uM CAP cannot induce Ca^{2+} transients in sensory neurons in the absence of extracellular Ca^{2+}. **B.** TRPV1 potentiation by ATP: the second CAP response is diminished compared to the first one (desensitization) and the third CAP response following ATP application is increased compared to the second one (potentiation). **C.** TRPV1 potentiation by ART: the second CAP response following 7-minute perfusion of ART-containing buffer is increased compared to the first one.

3.4.4 Data analysis

Amplitudes (ΔF) of Ca^{2+} increases caused by stimulation of neurons with KCl or CAP were measured by subtracting the baseline ratio of F_{340}/F_{380} from the peak F_{340}/F_{380} achieved on exposure to KCl or CAP. Latencies to maximal response (T_{max}) were measured between the time point of treatment and the one to reach the peak. Half-decay time ($T_{1/2}$) represented the time interval in the declining phase between the peak and the half of peak ratio (**Figure 6.2A**). The first CAP responses were compared between young and old neurons. Tachyphylaxis was measured by comparing the amplitudes of the first two CAP responses. Potentiation was analyzed by comparing the amplitudes of CAP responses before and after ATP or ART application. All values were represented as mean ± SE. Student's *t* test and ANOVA were used for most comparisons except that specific tests were indicated.

IV. RESULTS (1)
EFFECT OF AGING ON NORMAL PAIN SENSATION

In this chapter we investigated whether an age-related decline in afferent sensitivity was correlated with altered expression of membrane channel proteins. It is clear that afferent sensitivity is highly dependent on the expression of several classes of membrane channel proteins that regulate ion flow in response to a given stimulus. However, whether age-related changes in expression and/or function of ion channels contribute to altered neuronal sensitivity and excitability with aging is not known. To test whether changes occur in channel expression and distribution in the aging sensory system, we compared expression of receptors and channels, including the thermosensitive TRPV ion channels and the TTX-resistant sodium channels, in ganglia and nerves of young, middle-aged and old mice. These channels are involved in the generation and transmission of impulse trains in response to noxious stimuli. The relative expression level of receptor proteins particularly in GDNF family was also assayed to examine the relationship between trophic factor signaling and measured neuronal properties. We found reduced expression of TRPV1, Nav1.8 and other channels/receptors in the aging PNS, which correlated very well with a decrease in heat sensitivity in aged mice.

4.1 DECREASED THERMAL SENSITIVITY IN AGED MICE

We began our analysis of age-related changes in sensory neurons by comparing the behavioral response properties of young and old male and female C57Blk6 mice (**Figure 4.1**). Measures of response latency to an applied noxious thermal stimulus showed that, for both males and females
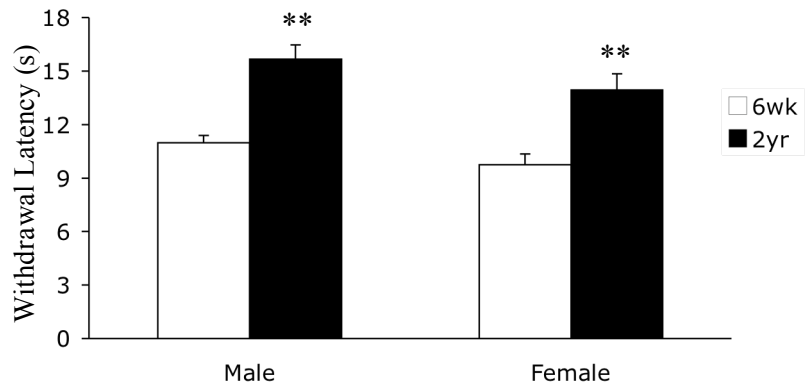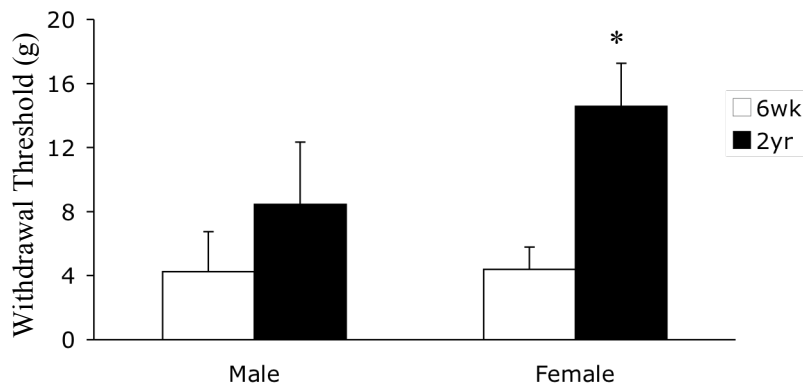
Figure 4.1 Thermal and mechanical sensitivity in young and old mice. **A**. Thermal sensitivity was decreased in both male and female 2-year old mice compared to 6-week old mice of the same sex. **B**. Mechanical sensitivity was decreased in 2-year old female mice but was not affected in aged male mice compared to young mice of the same sex. * - $P < 0.05$, ** - $P < 0.01$.

(n = 11–12 animals/group), a longer latency occurred in 2-year old animals (**Figure 4.1A**). Comparison of all females (young and old) to all males (young and old) showed that as a group, females had a shorter latency than males, i.e., they were more sensitive to thermal stimuli ($P < 0.05$). In later CFA studies middle-aged (16-month old) male mice also showed a longer latency compared with 2-month old male mice (10.4 ± 1.3s vs. 6.0 ± 0.7s, $P < 0.01$, n = 12). To determine if differences in mechanical sensitivity are present in aged animals, von Frey filaments with varying thickness were applied to the hind-paw and the amount of force that evoked a response (lifting or licking) recorded. Although no significant difference was measured between young and old male mice, repeated tests indicated that aged female mice tended to have a higher mechanical threshold (Komolgorov–Smirnov test, $P < 0.05$, n = 5–6 per group) (**Figure 4.1B**). Thus, using the described assays, 24-month old female mice seemed less sensitive to both thermal and mechanical stimuli whereas aged males were only less sensitive to heat stimulation.

4.2 REDUCED VOLTAGE-GATED CHANNELS IN DRG OF AGED MICE

Voltage-gated sodium channels are responsible for the rising phase of the action potential (AP) and play a key role, with potassium channels, in determining the excitability of sensory neurons. The neuron-specific sodium channels Nav1.8 and Nav1.9 are predominantly expressed in nociceptors, many of which are heat sensitive. To determine whether Nav1.8 and Nav1.9 expression are altered in aging sensory neurons, we first examined the abundance of the mRNA encoding each gene using reverse transcriptase-PCR assays. In pooled RNA from cervical, thoracic and lumbar ganglia, a slight reduction (18%; $P < 0.05$) in Nav1.9 mRNA was measured in DRG from 2-year old animals (n = 5 / age group) (**Table 4.1**). However, the level of Nav1.8 transcript was reduced 43% in ganglia from 2-year old mice (**Figure 4.2A** and **C**). To examine expression on the protein level, proteins extracted from pooled and lumbar ganglia were analyzed using western immunoblotting with an antibody made to the rat Nav1.8 channel. A significant 37% decrease in Nav1.8 abundance was detected in pooled DRG (**Figure 4.2B** and **C**). Reduced Nav1.8 protein was also found in pooled lumbar DRG of both aged and middle-aged mice (**Figure 4.2B)**, though Nav1.8 mRNA did not change in old lumbar DRG (**Table 4.1**).

Gene expression of voltage-gated calcium channel Cav2.2, which is involved in neurotransmission in the nociceptive pathway, was also decreased 32% in lumbar DRG from middle-aged animals (n = 4 / group) (**Table 4.1**). These findings are consistent with decreased electro-excitability in aged DRG neurons reported in other studies (Scott et al., 1988). Interestingly, a 22% increase in expression of substance P, a neuropeptide involved in signaling processing of nociception (DeVane, 2001), was measured in DRG from old mice (n = 5/group) (**Table 4.1**). This expression may compensate in part for the putative decrease in neurotransmission during aging.

Table 4.1 Gene expression in DRG and skin of aged mice compared to young animals. The relative expression of mRNAs was measured using Reverse transcriptase-PCR assays.

Class/function	Gene assayed	L2~5DRG	Pooled DRG	Footpad skin
Channel	Nav1.8	n.c.	↓43% *	
	Nav1.9	n.d.	↓18% *	
	Cav2.2	↓32%*	n.d.	
Neurotransmitter	Substance P	n.d.	↑22% **	
GFL receptor	GFRα2	↓13% *	n.c.	
	GFRα3	↓32% *	↓29% *	
	RET	↑35% **	n.c.	
Neurotrophic factor	Artemin	n.d.	↑122% *	n.c.
	NGF	n.d.	↑85% **	n.c.
	GDNF	n.d.	n.c.	↑55% **
Glial cell expression	ETRB	↓53% *	↓37% **	
	Myelin	n.d.	↓27% **	
Inflammation	IL-6	↑162%**	n.d.	n.c.
	LIF	n.d.	n.d.	↓33%*

n.d. - not done, n.c.- no change, * - $P < 0.05$, ** - $P < 0.01$
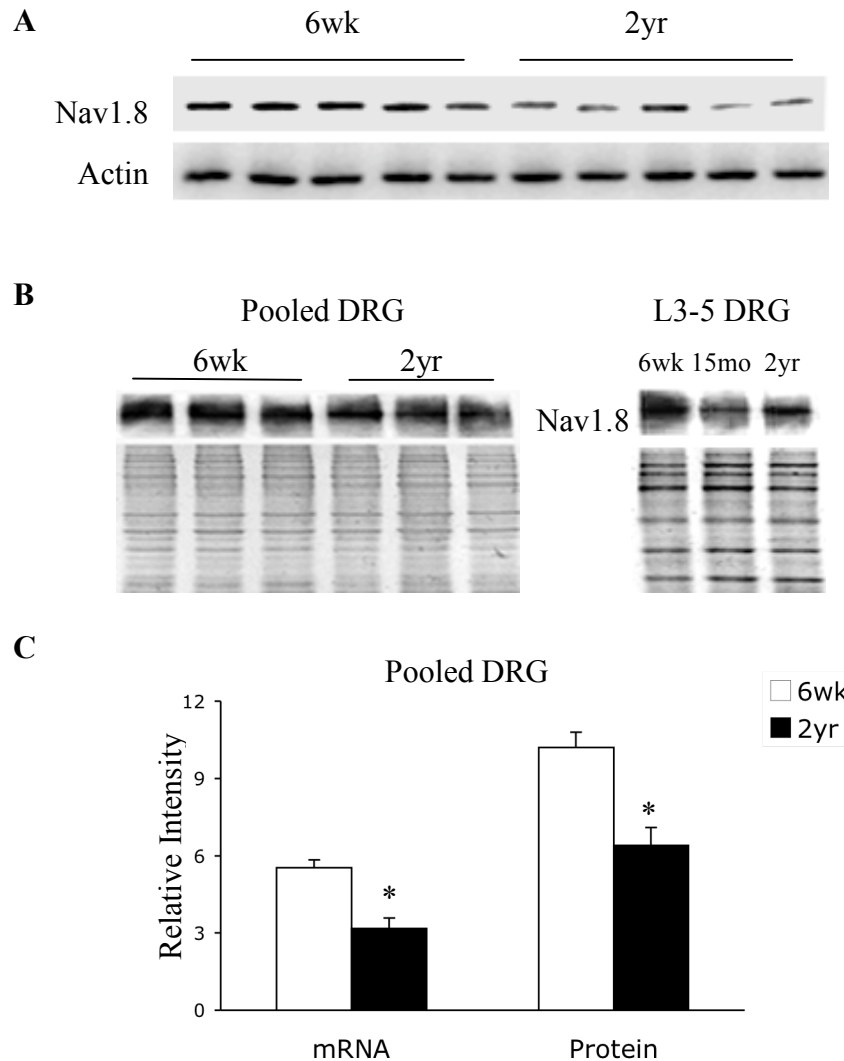
Figure 4.2 Decreased expression of Nav1.8 in DRG of old male mice. **A.** Abundance of Nav1.8 mRNA determined using ^{32}P-dCTP spiked RT-PCR assasys was found to be decreased in pooled DRG from 2-year old mice compared to 6-week old mice (n = 5). Actin was used as a control for the reaction and gel loading. **B.** Relative amount of Nav1.8 protein measured using western blotting was also decreased in pooled DRG (n = 3) and pooled lumbar 3-5 DRG (from 3 animals each age group) of aged mice. Coomassie stained gel of DRG samples is shown beneath blot to show sample loading. **C.** Relative intensity of Nav1.8 mRNA and protein bands in pooled DRG was measured. The mean intensity level of mRNA and protein was decreased in aged DRG by 43% and 37% compared to samples from young DRG, respectively. * - $P < 0.05$

4.3 REDUCED TRPV1 PROTEIN IN THE AGED PNS

The reduced behavioral sensitivity exhibited by middle-aged and aged Blk6 mice indicated that aged animals are less sensitive to thermal stimuli applied to the foot. To begin to identify the cellular level changes that could underlie this reduction in thermal sensitivity, we analyzed the expression of genes in the thermosensitive TRP channel family in young and aged DRG. We focused on the TRPV (TRPV1, TRPV2, TRPV3 and TRPV4) channels that are known to be heat responsive. Expression in pooled ganglia from the L3/L4/L5 lumbar ganglia was first analyzed using RT-PCR. Only lumbar ganglia were analyzed in order to enrich for neurons that innervate the hind-foot, which is the site of behavioral testing. Both pooled and lumbar DRG samples showed no difference in TRP expression on the transcriptional level (data not shown). To determine whether an age-related reduction occurred for TRP channels on the translational level, we analyzed TRPV1 expression in protein extracts obtained from pooled DRG and lumbar DRG using western immunoblotting. Analysis of pooled DRG showed no statistically significant change in TRPV1 ($P = 0.06$), whereas in lumbar DRG, a reduction in TRPV1 in aging ganglia was present at 15 months of age that was further reduced in 2 yr-old ganglia (**Figure 4.3A**). These results suggest that the amount of TRPV1 protein steadily declines in aged neurons despite the lack of change on the RNA level. In addition, this decline may be greater in neurons that project to distal targets in the leg and foot. To examine TRPV1 expression on a per cell level, L4/L5 DRG were immunolabeled with TRPV1 antibody to determine the percentage of neurons that express TRPV1 in young and aged ganglia (**Figure 4.3B**). The percentage of TRPV1-positive neurons in DRG from 2–3-month old mice was not different from the percent of TRPV1-positive neurons in 2-year old ganglia (young, $51.4 \pm 5\%$ versus old, $50.3 \pm 5.8\%$). Coupled with the reduction of TRPV1 protein detected by immunoblotting, these data suggest that on a per cell level, the decrease in TRPV1 protein may be related to translational processing.

The reduced level of TRPV1 protein in DRG of aged mice led us to ask whether the terminals of these sensory neurons would also exhibit deficiency in TRPV1 level. Tibial nerves from young and old animals were immunolabeled using the anti-TRPV1 antibody to test this possibility. Immunolabeling showed an apparent reduction in the number of TRPV1 positive fibers in nerves from aged animals (**Figure 4.4C**). To verify this decrease we isolated protein

A

Pooled DRG

L3-5 DRG

6wk   2yr

6wk 15mo 2yr

TRPV1

B

6wk

2yr
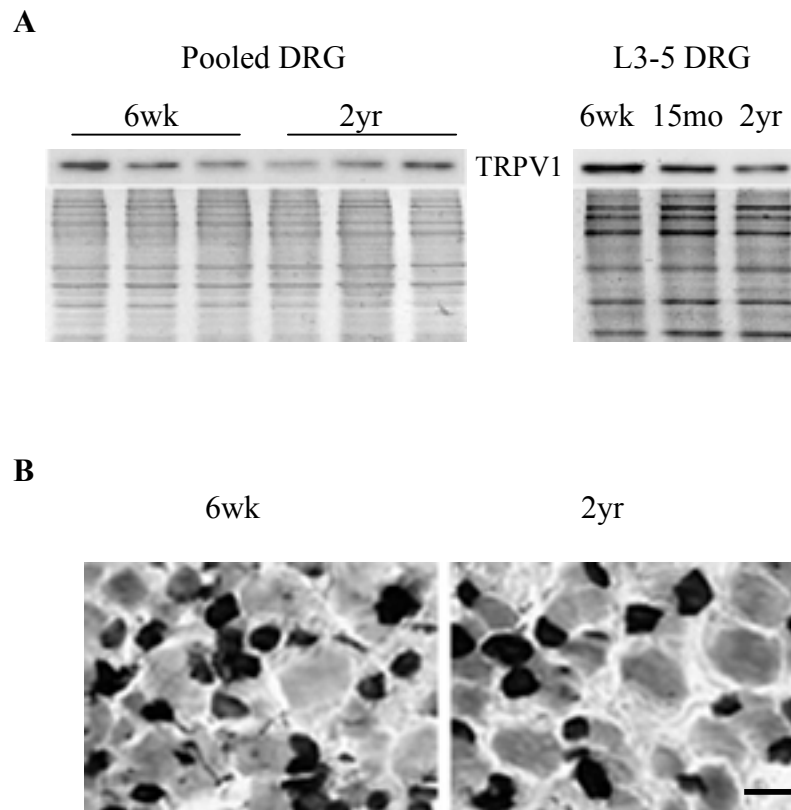
Figure 4.3 Expression of TRPV1 in DRG of young and aged mice. **A.** Relative amount of TRPV1 protein in pooled DRG (n = 3/group) and lumbar (L3~5) DRG of young and old animals was determined using western blotting. No significant decrease in TRPV1 level in pooled DRG was found. A decrease in lumbar DRG pooled from 3 animals (9 animals total) was detected in 15month-old (9%) and 2year-old animals (27%) when compared to 6 week-old mice. Coomassie stained gel of DRG samples is shown beneath blot to show loading. **B.** TRPV1 immunolabeling of L4/5 DRG of young and old mice shows prominent expression in smaller neurons. Lighter labeling was also found in some neurons. Both types of labeled cells were counted as TRPV1-positive. The percentage of TRPV1-labeled neurons was 51% ± 5% in young L4/5 DRG and 50% ± 6% in old DRG, and does not change with age. Bar in B = 50μM
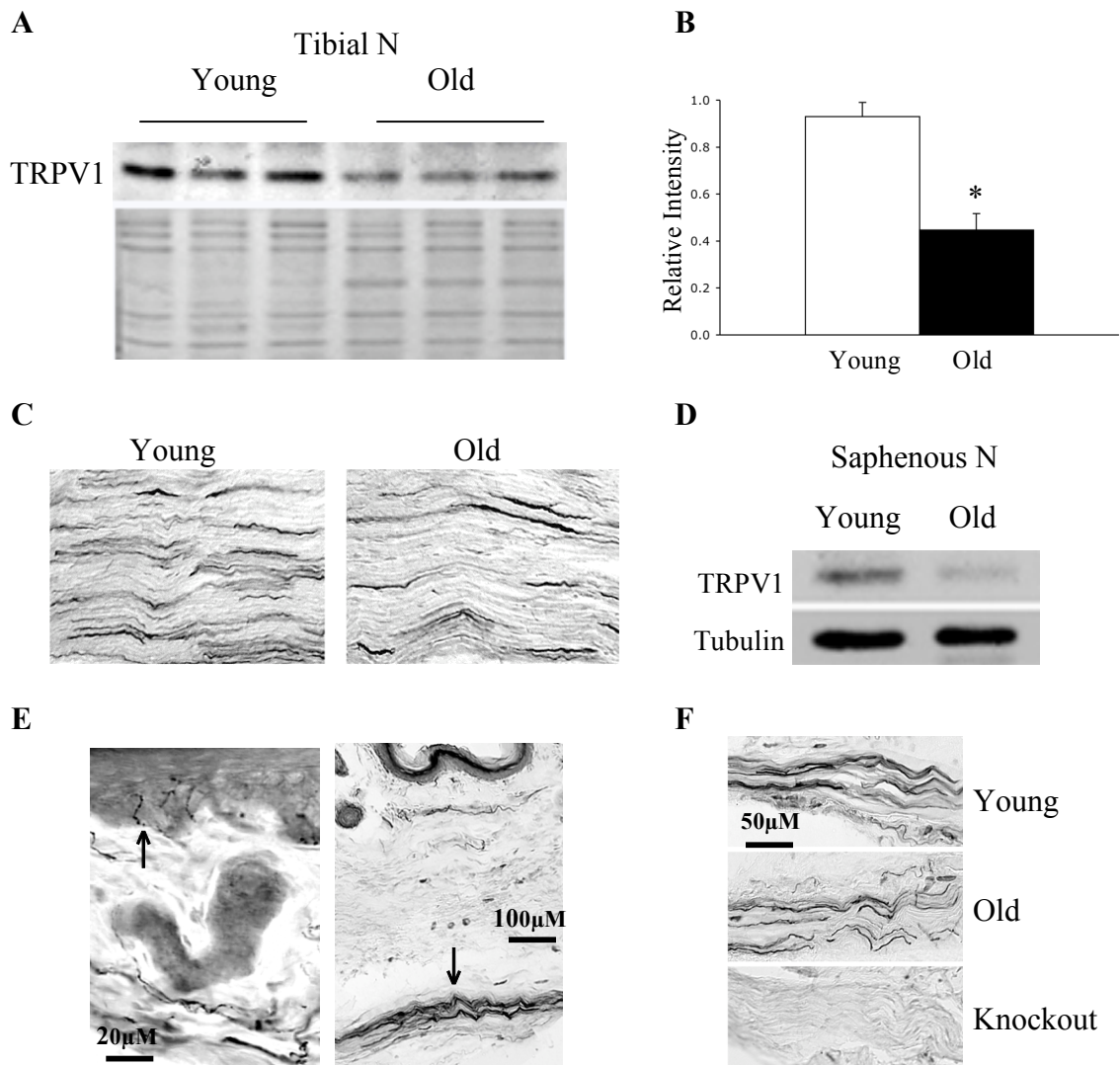
Figure 4.4 Decreased TRPV1 in peripheral nerves of aged mice. **A.** Western blot of TRPV1 protein in tibial nerves of young and old animals (n = 3/group) show TRPV1 reduction in aged samples. **B.** Plot of band intensity measured from immunoblot shown in A. **C.** Immunolabeling of sections of tibial nerve from young and aged animals. A reduction of TRPV1 fibers appears in nerve from old animals. **D.** Western blot also shows decreased TRPV1 in aged cutaneous saphenous nerves. Each lane represents a sample pooled from 3 young and 3 aged mice. **E.** TRPV1 fibers in epidermis (left arrow) and nerve bundles in deep dermis (right arrow) of plantar skin. **F.** TRPV1 fibers in dermal nerve bundles were compared in young and aged mice. Note reduction in TRPV1 labeling in aged mice and no labeling in nerves of TRPV1 knockout mice.

from the tibial and saphenous nerve. Whereas the tibial nerve primarily contains nerve fibers innervating muscle and skin, the saphenous nerve is a purely cutaneous nerve that innervates the skin of the leg and foot. A significant reduction in the level of TRPV1 protein in tibial (52%, $P < 0.05$) and saphenous nerve samples (25%,) was measured (**Figure 4.4A, B** and **D**). To determine if TRPV1 was reduced in sensory afferents in the skin of aged animals, we compared the density of TRPV1-positive fibers in the footpad of young and aged animals. Although the anatomical variation in the footpad hindered the quantitative measure of TRPV1-positive fibers, a general decrease in TRPV1-positive fibers was apparent when comparing the overall number of TRPV1-positive fibers coursing through the nerve bundles in the deep dermal tissue of the foot (**Figure 4.4E** and **F**). TRPV1 afferents appeared fewer in the aged animals, which is consistent with the reduction in TRPV1-labeled fibers in the tibial and saphenous nerves.

4.4 REDUCED GFRα3 RECEPTOR IN AGED SENSORY GANGLIA

Accumulating evidence suggests that the maintenance and sensitivity of sensory neurons is modulated by the level of trophic support provided by cells in peripheral and ganglionic tissues. This support appears to decline in aging systems, as evidenced by the reduction in mRNAs encoding the Trk neurotrophin receptors in sensory neurons of the aging rat (Bergman et al., 1996; Bergman et al., 1999a). A decline in growth factor support and signaling may impede synthesis and transport of neuron specific proteins (e.g., TRPV1 channels) and thereby reduce neuronal sensitivity, leading to higher response thresholds to thermal stimuli. With this possibility in mind, we examined expression of receptors for artemin, a neurotrophic factor that supports a nociceptor neuron population that expresses high levels of TRPV1. We examined the relative expression of the artemin specific GPI-linked binding component GFRα3 and its associated signaling component, the tyrosine kinase receptor RET in lumbar DRG using RT-PCR and immunoblot assays (**Table 4.1**). Relative to young animals, GFRα3 mRNA was reduced 32% in lumbar DRG in 15-month old animals. Protein level expression of GFRα3 was also reduced as shown by immunoblot assay of lumbar DRG pooled from three animals of each

A

6wk 15mo 2yr

GFRα3

B

C

Young   Old
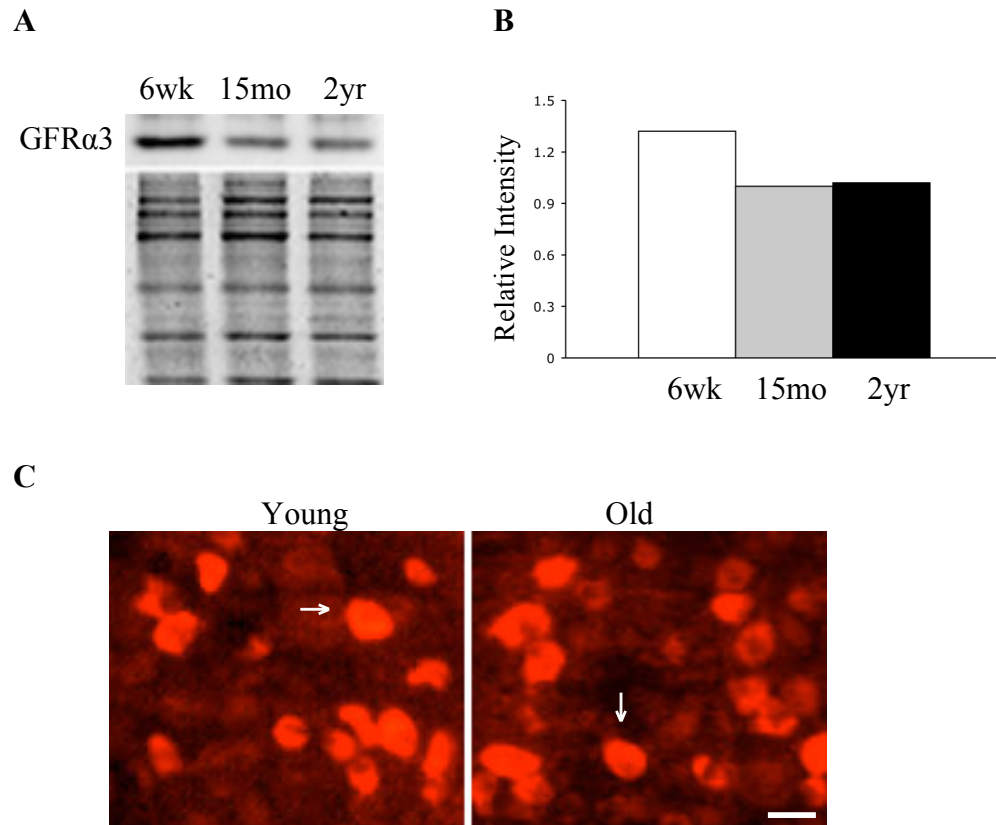
Figure 4.5 Expression of GFRα3 receptor in lumbar DRG of young and old mice. **A.** Western blot of protein extracted from lumbar DRG indicates reduction of GRFα3 in aging animals. Each lane represents protein pooled from three animals at each age analyzed. **B.** Plot of band intensity measured from immunoblot shown in A. The amount of GFRα3 decreased 24% and 23% in 15-month and 2-year age group, respectively. **C.** GFRα3 immunolabeling of L4/5 DRG of young and old mice shows prominent expression in smaller neurons. Lighter labeling was also found in some neurons. Both types of labeled cells were counted as GFRα3-positive. The percentage of GFRα3-labeled neurons was 31% ± 2.6% in young L4/5 DRG and 28% ± 0.7% in old DRG, and does not change with age. Bar in B = 50μM

age group (total of nine animals) (**Figure 4.5A** and **B**). A 24% reduction was measured in the level of GFRα3 protein in the lumbar ganglia of 15-month old animals relative to 6-week old animals. Thus both transcriptional and translational down-regulation of GFRα3 occurred in aged ganglia. However, the percentage of GFRα3-positive neurons in lumbar DRG from 2–3-month old mice was not different from the percentage in 16-month old ganglia (young, 31% ± 2.6%; old, 28% ± 0.7%) (**Figure 4.5C**), indicating that there was no loss in GFRα3-positive neurons in DRG of aged mice. We also examined the relative expression level of other GDNF-ligand binding molecules. Measure of GFRα1, which binds GDNF, showed no significant change in expression in aging DRG (contrary to the increase found in aging rats), whereas GFRα2, which binds neurturin, was slightly decreased (13%, $P < 0.05$). Interestingly, the RET tyrosine kinase receptor, which is the signaling component for all GDNF-family ligands, was increased 35% in the aged DRG ($P < 0.01$) (**Table 4.1**).

4.5 AGE-REGULATED GENES IN SENSORY GANGLIA AND SKIN

To determine whether the age-induced decrease in TRPV1 and GFRα3 expression in the cutaneous sensory system correlates with a change in neurotrophic factor expression, we measured mRNAs encoding NGF, GDNF and ART in DRG and hind-paw skin using RT-PCR. In aged skin an increase in GDNF was detected but no change was found in ART or NGF mRNAs. In DRG of aging animals, expression of the ART and NGF growth factors increased while the level of GDNF mRNA was unchanged (**Table 4.1**). The NGF receptor TrkA and GDNF receptor GFRα1 were also unchanged (data not shown), although RET increased in the aged DRG. In contrast, expression of the GFRα3 receptor decreased in aged ganglia. To identify the source of the increased level of ART expression in the aging ganglia, we carried out immunolabeling of artemin in young and old TG and lumbar DRG. This analysis showed ART reactivity localized primarily in small support cells that encircle the sensory neurons (**Figure 4.6A**). Interestingly, ganglia of old mice exhibited a significantly greater level of artemin reactivity in these peri-neuronal support cells compared to ganglia from young animals (**Figure 4.6B** and **C**). This increase correlates with the increase in ART mRNA measured using RT-PCR.
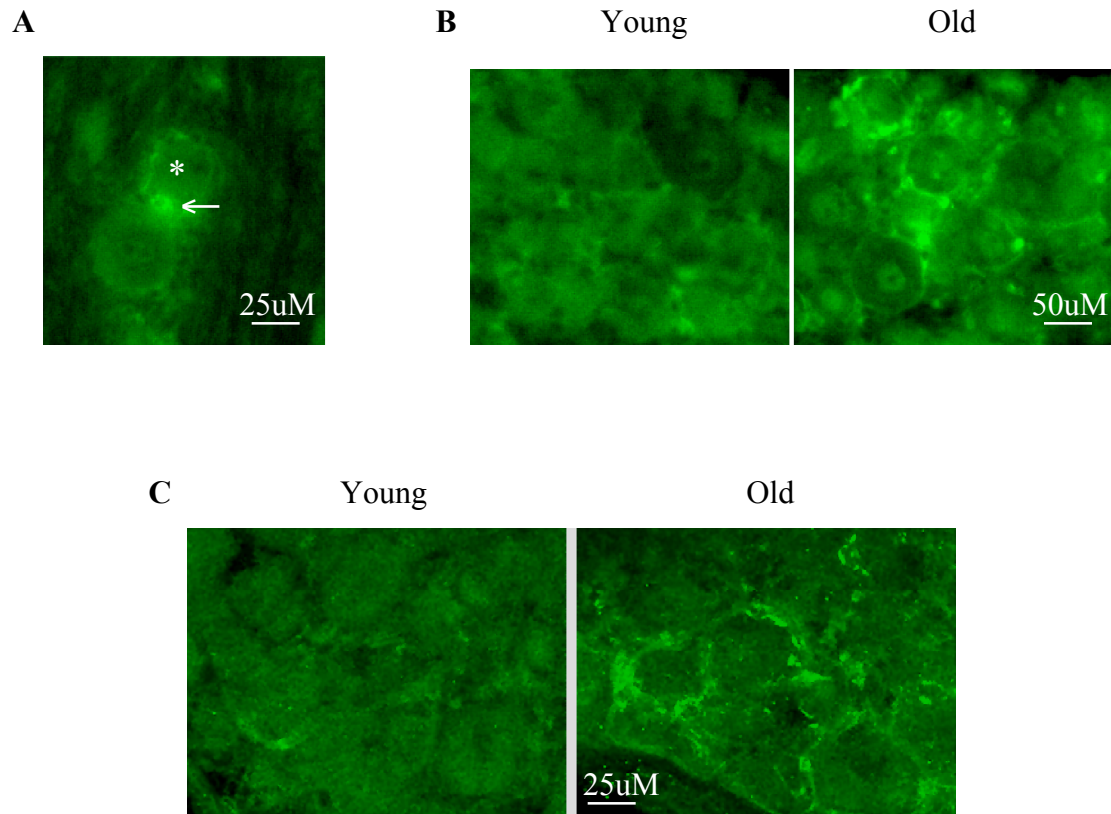
Figure 4.6 Expression of ART in sensory ganglia of young and old mice. **A.** Immunolabeling of ART in sensory ganglia shows that ART reactivity localizes primarily in small support cells (arrow) that encircle the sensory neurons (asterisk). **B.** In TG, old mice exhibite a significantly greater level of artemin reactivity in these peri-neuronal support cells compared to cells from young animals. **C.** Similar to TG, the level of ART immunoreactivity in these peri-neuronal support cells of lumbar DRG of old mice is also significantly higher compared to ganglia of young mice.

We further analyze gene expression in trigeminal sensory ganglia of 6wk-old and 2yr-old mice (n = 3/group) using Affymetrix microarrays. For this analysis, a regulated gene was defined as one with a fold difference significantly greater than 1.5 (P < 0.05) (Costigan et al., 2002). Seven of the total 16 changed genes in female (**Table 4.2**) and 6 of 21 regulated genes in male (**Table 4.3**) were immune/inflammation related, e.g., complement and lysozyme. Although the source of the elevated immune/inflammation-related transcripts in the aged ganglia is not yet defined, they may be from infiltrating macrophages and leukocytes. These cells are known to enter sensory ganglia, particularly following viral infection (Kodukula et al., 1999). Satellite cells and Schwann cells are also a possible source since they have been reported to synthesize inflammatory cytokines as well (Watkins and Maier, 2002). Microarray analysis also showed decreased expression of other genes such as endothelin receptor B (ETRB) and myelin (**Table 4.2**), which are expressed in satellite support cells and Schwann cells, respectively. The decrease in these transcripts was also found in the DRG (**Table 4.1**). The reduced expression of ETRB in support cells coupled with the increased expression of ART in these cells suggest that the functional properties of satellite cells changes in the aging system.

4.6 CONCLUSIONS

These studies examined changes in genes and/or proteins associated with nociception, neurotrophic support and inflammation in trigeminal ganglia and DRG of young and aged mice. We found a decrease in expression of channel proteins (TRPV1, Nav1.8 and Nav1.9, and Cav2.2) involved in detection and transmission of noxious stimuli in the aging PNS. This decrease correlated with impaired sensation of noxious heat measured in aged animals. Reduction of these channels in the PNS may be a consequence of decreased signaling through GFRα3 pathways, which may result from aging-induced degeneration of primary sensory neurons and/or neuronal-support cells. Increased expression of immune/inflammation genes in the PNS with aging suggests that age-related immune/inflammation responses may also have a critical role in age-induced neuronal degeneration in the peripheral nociceptive system.

Table 4.2 Regulated genes in trigeminal ganglia of 2 yr-old female mice compared with 6 wk-old female mice using microarray assay.

Class/function	Gene	Accession#	Fold change
Immune/	S100 calcium binding protein A8	M83218	3.6↑
Inflammation	Lysozyme	M21050	2.7↑
	S100 calcium binding protein A11	U41341	1.6↑
	S100 calcium binding protein A9	M83219	1.6↑
	Complement component 4	X06454	1.6↑
	Macrophage expressed gene 1	L20315	1.5↑
	Lymphocyte antigen 6 complex	U47737	1.5↓
ECM protein/	Procollagen, type I, alpha 1	U03419	1.9↓
Cytoskeleton	Procollagen, type III, alpha 1	X52046	1.8↓
Hormone/	Prolactin	X04418	8.5↑
Neuropeptide	Glycoprotein hormones	AV173687	3.1↑
	Tachykinin 1	D17584	1.5↑
Enzyme	UDP-glucuronosyltransferase 8	U48896	2.3↓
	ATPase aminophospholipid transporter	U75321	1.8↓
Glial expression	Proteolipid protein (myelin)	M16472	2.4↓
Unknown	H19 fetal liver mRNA	X58196	1.7↓

Table 4.3 Regulated genes in trigeminal ganglia of 2 yr-old male mice compared with 6 wk-old male mice using microarray assay.

Class/function	Gene	Accession#	Fold change
Immune/	Immunoglobulin kappa chain	M18237	3.4↑
Inflammation	Lysozyme	M21050	2.1↑
	P lysozyme structural	X51547	2.0↑
	S100 calcium binding protein A8	M83218	2.0↑
	Mus castaneus IgK chain gene	M80423	1.8↑
	Complement component 4	X06454	1.5↑
ECM protein/	Follistatin-related protein (TSC-36)	M91380	1.7↑
Cytoskeleton	Scgn10 like-protein	AF069708	1.5↑
	Reelin	U24703	1.5↑
	Microtubule-associated protein 4	M72414	1.5↑
	Procollagen, type I, alpha 1	U03419	2.5↓
	Procollagen, type I, alpha 2	X58251	2.1↓
	Procollagen, type III, alpha 1	X52046	2.1↓
	Procollagen, type IV, alpha 1	M15832	2.0↓
	Procollagen, type XV	AF011450	1.7↓
Protein/	Proteasome (prosome, macropain) subunit	AB003304	1.6↑
Lipid turnover	Apolipoprotein D	X82648	1.6↑
Glial expression	Proteolipid protein (myelin)	M16472	1.6↓
	Endothelin receptor type B	U32329	2.1↓
Unknown	H19 fetal liver mRNA	X58196	1.7↓
	Integral membrane protein 2A	L38971	1.6↓

V. RESULTS (2)
EFFECT OF AGING ON INFLAMMATION-INDUCED HYPERALGESIA

5.1 INTRODUCTION

Injury, infection or irritation of the skin, muscle or internal organs is often accompanied by inflammation related pain. An inflammatory state is recognized on the histological level by a large infiltration of the damaged tissue with various types of inflammatory cells that include neutrophils, macrophages and mast cells. These cells and cells at the site of injury release substances such as ATP, NGF and cytokines that can excite nociceptor neurons through activation of receptors on nerve terminals and in so doing, cause pain. Chronic, long-term release of inflammatory mediators can lead to changes in nociceptive pathways that may underlie persistent pain states. These changes may include abnormal expression of channel proteins and neuropeptide receptors on primary and secondary afferents. The most consistent reports about age differences in pain are the experience of acute pain related to specific pathological insults or infectious process. Clinical studies indicate that pain is more frequently absent in older patients with myocardial infarction, pneumothorax, and peptic ulcer diseases (Gibson and Helme, 2001; Moore and Clinch, 2004). Animal studies also have shown that inflammatory or neuropathic pain is modulated with aging in rat (Gagliese and Melzack, 1999, 2000). However, whether aging increases or decreases inflammatory pain responses is still controversial. Also undetermined is whether age-related differences in ion channel proteins contribute to different behavioral responses with age (Zhang et al., 2004; Kitagawa et al., 2005).

In this chapter we investigated whether aging modulated the response to noxious stimuli following CFA-induced peripheral inflammation, and whether this modulation was associated with age-related changes in channel proteins and neurotrophic signaling. Our results indicate that

inflammation-induced hyperalgesia was decreased in aged animals. This finding is consistent with a decrease in gene expression of the pain-related channels Nav1.8, TRPV1 and ASIC3 in aged animals during inflammation. In these studies we also measured the relative level of inflammation-associated transcripts. Age-related changes in gene expression of inflammatory cytokines IL-6 and leukemia inhibitory factor (LIF) correlated with changes in ART-GFRα3 expression. We postulate that under conditions of inflammation, expression of ART-GFRα3 regulates TRPV1 channel expression. These findings support the role of ART-GFRα3 signaling in age-induced functional deficits of the PNS.

5.2 AGED MICE EXHIBIT REDUCED THERMAL HYPERALGESIA

Prior to induction of inflammatory pain, mice were exposed to several days of pre-testing to determine an accurate baseline for each age group. Similar to our prior assay of behavior sensitivity, we found old mice less sensitive to noxious heat compared to young mice. Therefore a higher intensity of thermal stimulation (15%) was applied to aged animals compared with young animals (12%) to get an optimal baseline (young, 7.0 ± 0.6s; old, 7.8 ± 0.5s). At day 0, 12 young (8-week) and 12 old (16-month) mice were injected in the hind-paw with ~20ul of CFA. An expected peripheral edema in the hind-paw occurred following CFA injection that lasted for the 7 days of testing. In previous studies (Malin et al, unpublished) using young Blk6 male mice from Jackson Labs, CFA injection into the footpad caused thermal hyperalgesia that began within 1 day following injection and lasted for at least 3 days (**Figure 5.1A**). However, using young Blk6 mice from the NIA colony obtained from Harlan, thermal hyperalgesia was detected only on day 1 (4.3 ± 0.5s, $P = 0.002$) with full recovery by day 2 (**Figure 5.1B**). Also of interest was that no significant behavioral change was found in the aged mice from Harlan, although there was a trend toward a decrease in withdrawal latency on the first two days following CFA injection (day 1, 5.7 ± 1.1s; day 2, 6.1 ± 0.8s; $P = 0.086$) (**Figure 5.1B**). The short duration of hyperalgesia between the young (and lack of change in old mice) was surprising given that both sets of mice are of the Blk6 strain. These results suggested that environmental factors, e.g., colony conditions, baseline inflammation level, influenced the response to inflammatory stimuli.

A
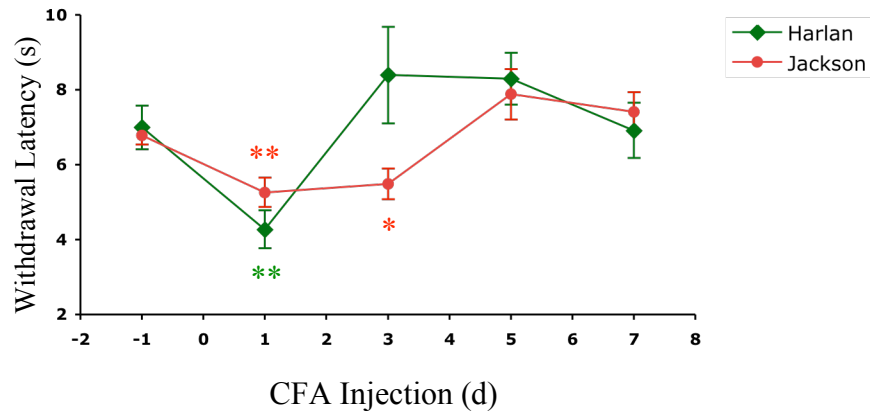
B
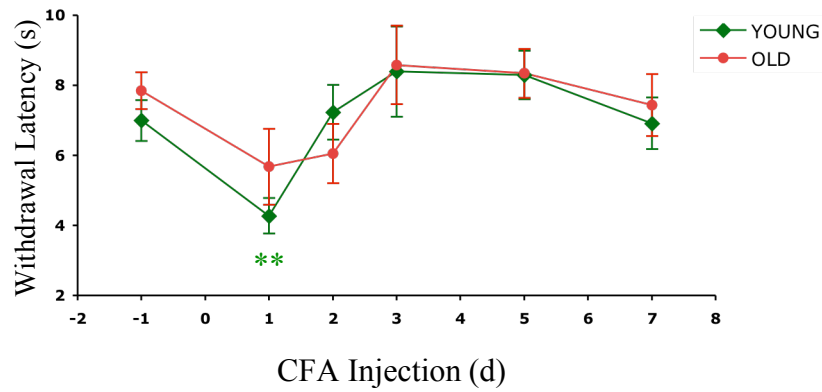
Figure 5.1 Thermal hyperalgesia induced by CFA injection. **A.** Thermal hyperalgesia, manifested by a shorter withdrawal latency post-CFA compared to pre-CFA, was compared between Harlan NIA (green) and Jackson lab (red) young Blk6 mice ($n = 12$/group). Thermal hyperalgesia lasted two days longer in Jackson mice compared to Harlan mice. **B.** Plots of thermal sensitivity in Harlan young (green) and old (red) Blk6 mice ($n = 12$/group). Thermal hyperalgesia was detected in young mice on day 1 post-CFA, but absent in old mice though withdrawal latency appears shorter on the first two days post-CFA. * - $P < 0.05$, ** - $P < 0.01$

51

In all three groups peak hyperalgesia occurred on 1 day post-injection. The greatest responses were detected in young mice from the Harlan colony, which showed a 39% decrease in withdrawal latency relative to baseline measure. Old mice from the Harlan colony and Jackson lab young mice only showed 27% and 22% decrease, respectively. Thus, CFA-induced inflammatory pain is of greater intensity and but shorter duration in young Harlan mice, but smaller and longer in old Harlan mice. These results suggest that aging results in decreased pain resulting from inflammation, which is consistent with clinical observations made in human.

5.3 CHANNEL/RECEPTOR EXPRESSION IN THE PNS POST-CFA

To identify whether abnormal expression of channels and receptors in the PNS associated with heat hyperalgesia induced by inflammation, we assayed relative levels of mRNAs encoding TRPV1, TRPV2, ASIC1, 2 and 3, and Nav1.8 in lumbar DRG after CFA injection. These channels are predicted to be involved in thermal, mechanical or chemical nociception and our previous screening showed some were decreased in the aged PNS. No change was measured in ASIC1, ASIC2 and TRPV2 mRNAs within 7 days post-CFA in either of the two age groups (data not shown). Nav1.8 expression decreased after 1d in old DRG though no significant change occurred in young DRG until 5 days post-CFA (**Figure 5.2A**). ASIC3, which is predominantly expressed in sensory neurons and is thought to mediate normal touch and pain sensation (Waldmann et al., 1997; Price et al., 2001), was decreased for 5 days after CFA in young animals while old animals did not show a change (**Figure 5.2B**). Although no difference was detected in the dynamic changes between young and old DRG after CFA, both Nav1.8 and ASIC3 mRNA levels in young DRG were significantly higher than in old DRG on day 1 post-CFA (**Figure 5.2A and B**). Similarly, TRPV1 expression did not change in both young and old animals, but the overall abundance of TRPV1 mRNA was significantly lower in old DRG than in young DRG following CFA injection (**Figure 5.2C**). GFRα3 expression was also lower in old DRG although it was decreased in both young and old DRG after inflammation (**Figure 5.2D**). We further examined the levels of GFRα3 and TRPV1 protein in the sciatic nerve of CFA-injected mice. Both GFRα3 and TRPV1 were decreased in young and old animals compared with
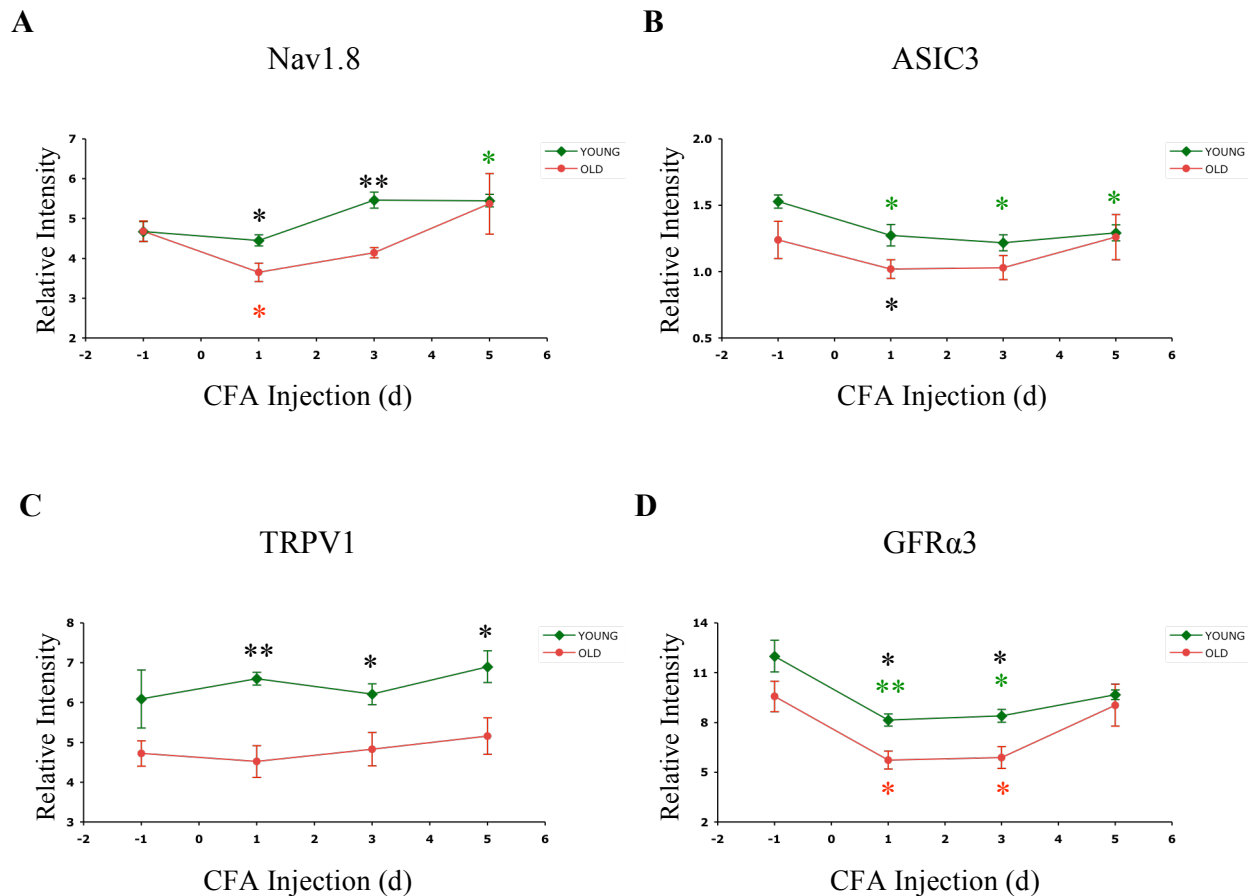
Figure 5.2 Relative mRNA expression in L2~5 DRG pre- and post-CFA injection (*n* = 4/group). **A.** Nav1.8 mRNA was increased from baseline level in young mice on day 5 (green asterisk) but decreased in old mice on day 1 (red asterisk) post-CFA, respectively. Note expression of Nav1.8 in DRG was higher than in old mice after CFA injection (black asterisks). **B.** ASIC3 was decreased in young mice (green asterisks) and not changed in old after CFA injection. **C.** No changes in TRPV1 expression were found in either young or old DRG before and after CFA injection. But the expression level was consistently higher in young DRG compared to DRG from old animals after CFA injection (black asterisks). **D**. GFRα3 expression was decreased in both young (green asterisks) and old (red asterisks) mice after CFA injection. A higher level was measured in DRG from young animals than in old (black asterisks) after CFA. * - *P* < 0.05, ** - *P* < 0.01
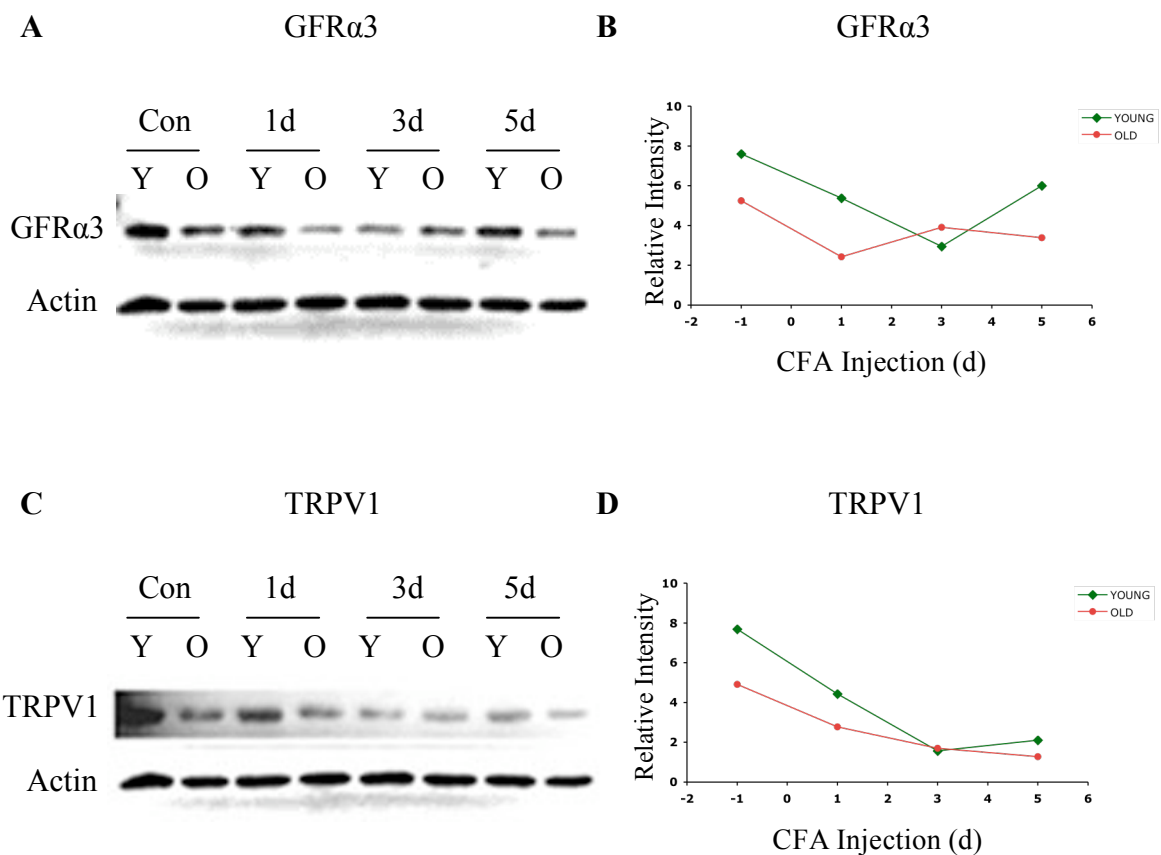
Figure 5.3 GFRα3 and TRPV1 protein in sciatic nerve pre- and post-CFA injection. **A.** Western blot of GFRα3 protein in sciatic nerve. GFRα3 was decreased in both young (Y) and old (O) mice on day 1, 3 and 5 (1d, 3d, 5d) after CFA injection when compared to pre-injection level (Con). Each lane represents a nerve sample pooled from 4 young or 4 aged mice. **B.** Plot of band intensity measured from immunoblot shown in A. **C.** Western blot of TRPV1 protein in sciatic nerve shows a reduction of TRPV1 in both young (Y) and old (O) mice after CFA injection (1d, 3d, 5d) compared to pre-injection (Con). Each lane represents a sample pooled from 4 young or aged mice. **D.** Plot of band intensity measured from immunoblot shown in C.

baseline levels. Consistent with the findings at the mRNA level, TRPV1 and GFRα3 protein was also lower in pooled sciatic nerve of old animals than in young mice (**Figure 5.3**). The finding that Nav1.8, ASIC3 and TRPV1 channel expression was generally lower in the PNS of old mice compared with young animals following CFA-induced inflammation supports the hypothesis that they are involved with the decreased inflammatory pain behavior exhibited by old animals.

5.4 GENE EXPRESSION OF ART AND NGF IN INFLAMED SKIN

Previous studies have shown that ART and NGF increase following CFA-induced inflammation in skin (Malin et al., 2006). ART and NGF are postulated to be important in signaling pathways that mediate inflammation-induced thermal hyperalgesia through their regulation of TRPV1 expression and activity. Given the linkage of these growth factors to pain signaling, we measured ART and NGF mRNA levels in hind-paw skin of young and old mice before and after CFA injection. We compared these measures to those made on the Blk6 mice obtained from the Jackson Laboratory. No significant difference was found in the baseline level of ART and NGF mRNA between young and old mice. However, ART mRNA level increased 2.5 fold in young mice ($P < 0.01$) and increased 3.9 fold in old mice ($P < 0.05$) on day 1 post-CFA injection, the day showing the peak behavioral response (**Figure 5.4B**). A decrease back to baseline in both young and old mice 3 days after CFA injection was then observed. In contrast to these changes, ART expression increased 10-fold ($P < 0.01$) in young mice from the Jackson lab on day 1 post-CFA and remained elevated to 4-fold higher ($P < 0.01$) on day 4 (**Figure 5.4A**). NGF mRNA level decreased 2-fold in both young and old Harlan animals only on day 1 ($P < 0.01$) (**Figure 5.4D**). In Jackson lab mice there was no change in NGF level in the CFA-injected hind-paw skin until day 7 after CFA (**Figure 5.4C**). These observations indicate that a dynamic change in ART and NGF mRNA level occurs in inflamed skin. The change in ART correlates with behavioral outcomes that follow CFA injection, suggesting that ART level in inflamed tissue may be more critical for inflammation-induced thermal hyperalgesia compared with NGF.
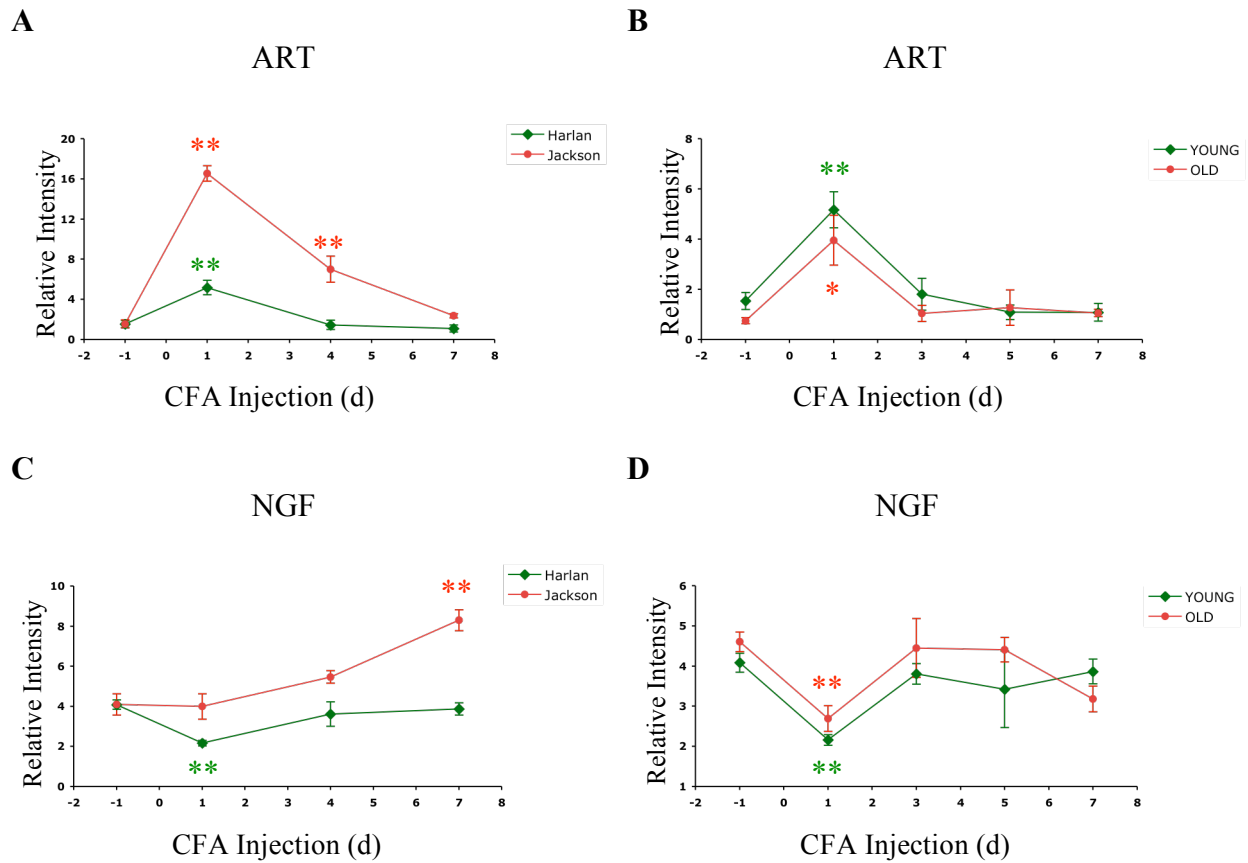
Figure 5.4 Relative expression of ART and NGF in inflamed footpad skin. **A.** ART expression was compared between young Harlan NIA (green) and Jackson (red) young Blk6 mice ($n = 4$/ group) before and after CFA injection. In Jackson mice ART increased 10-fold on day 1 and remained elevated at day 4 (red asterisks), only a 2.5-fold change was measured in Harlan NIA mice post-CFA. **B.** ART level was similar between Harlan young (green) and Harlan old (red) mice ($n = 4$/group) prior to CFA injection. A 2.5-fold increase in young skin (green asterisks) and a 3.9- fold increased in old skin (red asterisk), was found only on day 1 post-CFA. **C.** NGF expression in skin from Harlan NIA and Jackson young mice pre- and post-CFA injection ($n = 4$/ group). Note NGF was decreased on day 1 in Harlan mice (green asterisks) while increased on day 7 in Jackson mice (red asterisk). **D.** NGF expression in skin of Harlan young and old mice. A two-fold decrease was detected in both groups on day 1 post-CFA compared to pre-CFA ($n = 4$/ group) * - $P < 0.05$, ** - $P < 0.01$

5.5 GENE EXPRESSION OF LIF AND IL-6 IN INFLAMED SKIN

Inflammation is a complex set of responses to injury and infection. Cytokines are critical mediators in regulating a variety of cellular and molecular events during inflammation. Cytokines are a group of proteins with similar structure and share subunits in their receptor complexes, such as gp130, and have overlapping functions. Considering that CFA-induced changes in behavioral responses and expression of genes associated with nociception were different between young and old mice, and much more different between Jackson and Harlan NIA Blk6 mice, we hypothesized that differences in the immune system could underlie these outcomes. We therefore compared gene expression of two inflammatory cytokines, leukemia inhibitory factor (LIF) and interleukin 6 (IL-6), in hind-paw skin of the three groups of mice before and after CFA injection. IL-6 and LIF can act in both pro-inflammatory and anti-inflammatory ways, depending on inflammatory conditions (Gadient and Patterson, 1999). For example, during the acute phase reaction of inflammation, IL-6 can down-regulate the inflammatory response by suppressing pro-inflammatory IL-1 and TNF (Schindler et al., 1990). Both IL-6 and LIF have been found to play critical roles in inflammation-induced hyperalgesia (Banner et al., 1998; Opree and Kress, 2000).

In our studies no differences were found in the baseline level of IL-6 and ART mRNA in footpad skin of the three groups of mice (**Figure 5.5A** and **C**). However, the baseline level of LIF expression was 58% lower in Jackson mice and 33% lower in Harlan old mice when compared to it's level in young mice from the Harlan NIA colony ($P < 0.05$) (**Figure 5.5B**). In addition, there was no remarkable change in LIF mRNA in either group until day 4 after CFA injection (**Figure 5.6C** and **D**). In contrast to LIF mRNA, IL-6 expression dramatically increased 53-fold in Harlan young mice ($P < 0.01$), 44-fold in Harlan old mice ($P < 0.05$), and only 13-fold in Jackson mice ($P = 0.06$) on day 1 post-CFA (**Figure 5.6A** and **B**). Further regression analysis was done to identify whether these differences in cytokine level in footpad skin correlated with differences in ART expression and behavioral responses before and after CFA injection. This analysis showed that there was a linear relationship between baseline level of LIF mRNA in footpad skin and days of thermal hyperalgesia ($P = 0.05$) and a lower level of LIF correlated with longer hyperalgesia (**Figure 5.7B**). A linear relationship between fold-change of IL-6 and ART
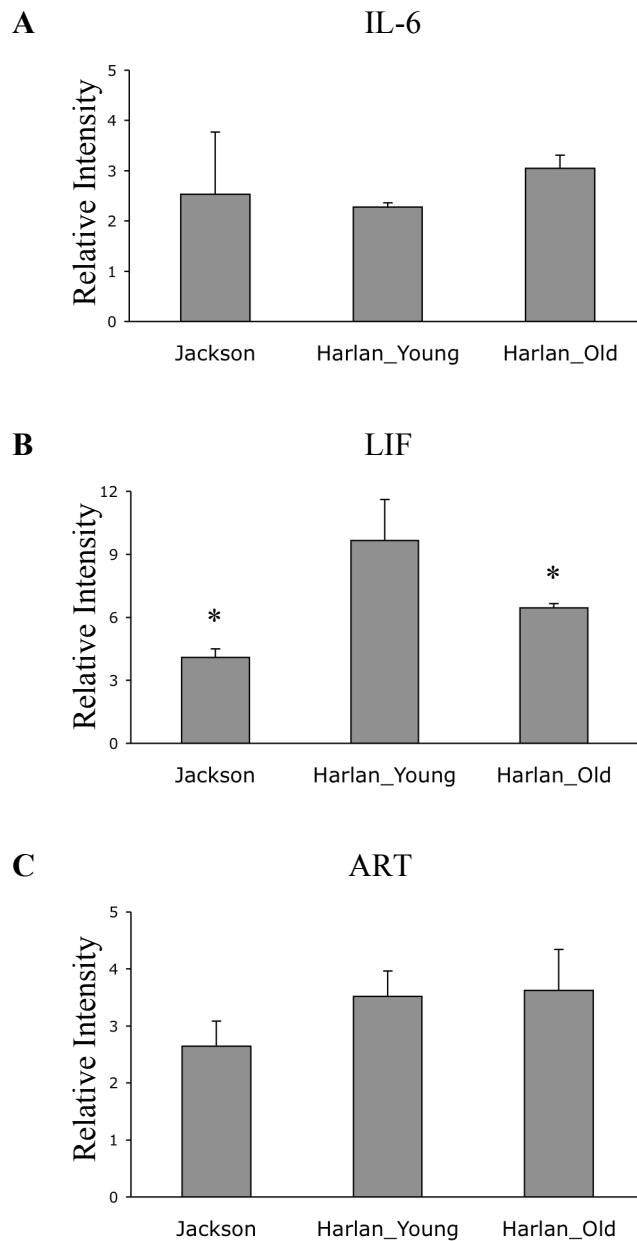
Figure 5.5 IL-6, LIF and ART mRNAs in footpad skin of naïve animals. **A**. No difference was detected in IL-6 expression in footpad skin among Jackson, young Harlan and old Harlan mice ($n = 4$ / group). **B.** LIF expression was decreased 58% in Jackson and 33% in Harlan old mice compared to Harlan young mice. **C.** No difference was found in ART expression among these three groups. * - $P < 0.05$

A IL-6

B IL-6

C LIF
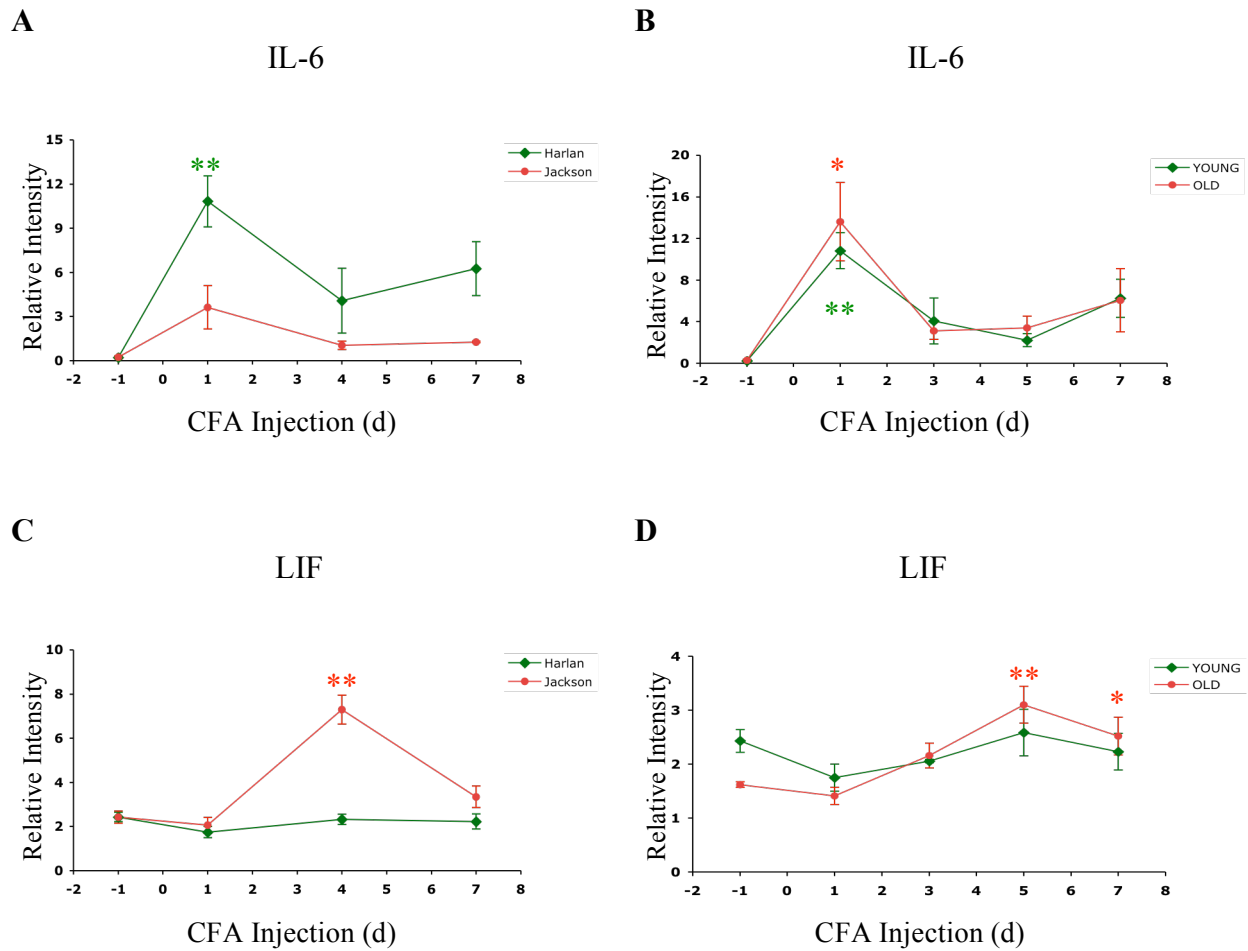
D LIF

Figure 5.6 Relative mRNA levels of IL-6 and LIF in inflamed footpad skin. **A.** IL-6 expression in young Harlan NIA and Jackson mice (*n* = 4/ group) before and after CFA injection. IL-6 mRNA in skin was increased 13-fold in Jackson and a 53-fold increase in Harlan mice on day 1 post-CFA. **B.** IL-6 expression was similar between Harlan young and old mice (*n* = 4/ group), and a 53-fold increase in young (green asterisks) and a 44-fold increase in old mice (red asterisk) were found on day 1 post-CFA compared to pre-CFA. **C.** LIF expression in Harlan and Jackson young mice pre- and post-CFA injection (*n* = 4/ group). Note LIF was increased on day 4 in Jackson mice (red asterisk) but no change was found in Harlan mice. **D.** LIF expression in Harlan young and old mice. An increase in LIF was only detected in old mice (red asterisks) post-CFA compared to pre-CFA (*n* = 4/ group) * - *P* < 0.05, ** - *P* < 0.01

A

ART vs. IL-6 at CFA 1d
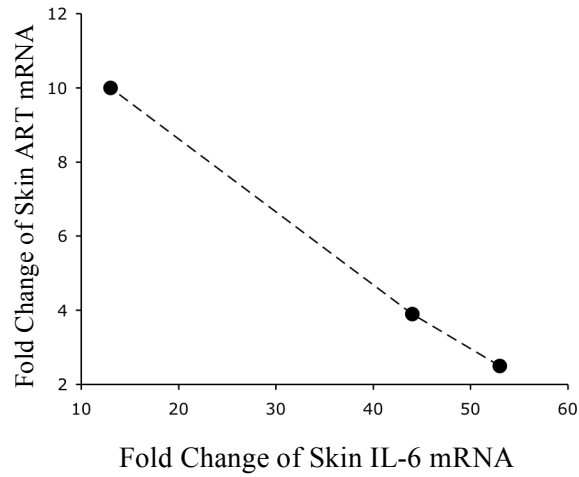
B

Days of thermal hyperalgesia vs. baseline LIF
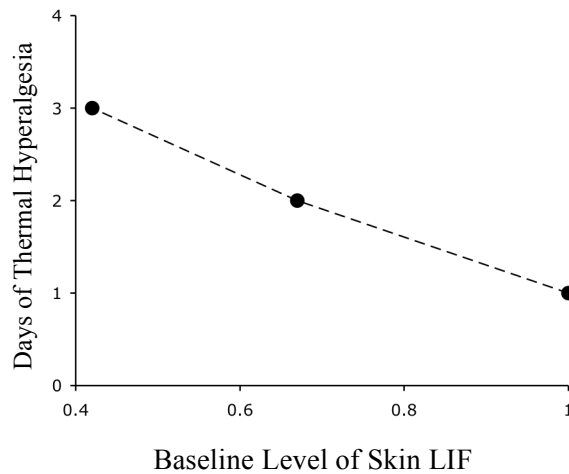
Figure 5.7 Correlation of IL-6 with ART and LIF with days of hyperalgesia **A**. On day 1 post-CFA the fold change of IL- 6 expression in inflamed skin linearly correlated with the change of ART expression (ART = 12.429 − 0.19 * IL-6, $P < 0.05$). **B**. The low baseline level of skin LIF expression was closely correlated with the 3-day thermal hyperalgesia induced by CFA in Jackson Blk6 mice (Days = 4.387 − 3.427 * LIF, $P = 0.05$).

expression on day 1 after CFA with higher IL-6 increase related to lower ART increase (**Figure 5.7A**), was also determined ($P < 0.05$). These findings suggest that LIF and IL-6 may have anti-inflammatory effects manifested by shorter thermal hyperalgesia. Reduced hyperalgesia is related to higher baseline level of LIF and by lower ART expression and correlated with a higher level of IL-6 mRNA during CFA-induced inflammation.

5.6 CONCLUSIONS

To address the question of whether age modulates the degree of pathological pain and whether modulation of normal pain sensitivity by aging is involved in age differences in pathological pain, we studied thermal hyperalgesia in mice with CFA-induced peripheral inflammation. We found that age not only decreased normal pain sensitivity but also inflammation-induced thermal hyperalgesia. Reduced behavioral responses to noxious heat are consistent with the molecular findings of decreased TRPV1 mRNA and protein in the PNS of aged animals following inflammation, which may result from decreased ART-GFRα3 signaling. These results also suggest that ART-GFRα3 signaling has a critical role in inflammatory pain. Preliminary studies also indicate that LIF and IL-6 act as anti-inflammatory factors that can regulate sensitivity to inflammatory pain, perhaps in conjunction with ART-GFRα3 signaling. Differences in behavioral sensitivity to inflammatory pain in Blk6 mouse line was also revealed and may be related to the "inflammatory tone" of the animals that is present prior to CFA injection. These findings strongly suggest that inflammation may be involved in the aging process of the PNS via effects on GFRα3-dependent neurotrophic signaling.

VI. RESULTS (3)

EFFECTS OF ARTEMIN ON TRPV1-DEPENDENT RESPONSES IN DRG NEURONS

Findings from our *in vivo* studies indicate involvement of ART-GFRα3 signaling in the aging process of the PNS. Previous studies have shown that ART/GFRα3 signaling modulates TRPV1 expression and function. Therefore we investigated whether ART regulated TRPV1 function in aging neurons using calcium imaging. Resting DRG neurons typically have very low membrane permeability to Ca^{2+} in spite of a huge electrochemical driving force in favor of Ca^{2+} influx. The TRPV1 channel has much higher permeability to Ca^{2+} than other ions like Na^+ and K^+. Thus activation of TRPV1 induces large Ca^{2+} influx, and in turn results in rapid changes in intracellular Ca^{2+} concentration that can be detected using fluorescent Ca^{2+}-binding indicators. These indicators are excited at slightly longer wavelengths in a Ca^{2+}-free form than in a Ca^{2+}-bound form. By measuring the ratio of fluorescence intensity at two excitation wavelengths, the concentration ratio of the Ca^{2+}-bound indicator to the Ca^{2+}-free can be determined, and then an accurate free Ca^{2+} concentration can be measured. Entering cells by diffusion, these indicators make it possible to monitor Ca^{2+} flux in a large number of individual cells simultaneously using a fluorescence microscope (Alberts *et al.*, 1994). Though this approach lacks specificity, Ca^{2+} imaging is a helpful technique to assess activities of membrane Ca^{2+}-permeable channels.

6.1 INTRODUCTION

Activation of membrane voltage-gated calcium channels (VGCCs) or TRPV1 channels results in changes of intracellular free Ca^{2+} concentration and shape the intracellular Ca^{2+} transients.

Calcium movement reflects three major processes (Dedov and Roufogalis, 2000): entry of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels and release of Ca^{2+} from the internal Ca^{2+} stores of ER, intracellular Ca^{2+} uptake by ER and mitochondria, and extrusion of Ca^{2+} by plasma membrane ATP-dependent Ca^{2+} pumps and the Na^{+}/Ca^{2+} exchangers. When the rate of Ca^{2+} influx and release is greater than the rate of Ca^{2+} uptake and efflux, the intracellular free Ca^{2+} concentration increases and shapes the rising phase of the Ca^{2+} transients; when the rate of Ca^{2+} influx and release is slower than the rate of Ca^{2+} uptake and efflux, the intracellular free Ca^{2+} concentration decreases and is represented by the declining phase of the Ca^{2+} transients; and the amplitude represents the maximal intracellular Ca^{2+} load. Analysis of the Ca^{2+} transients initiated by activation of plasma membrane Ca^{2+} channels alone provides indirect evidence about functional properties of these channels.

An age-related difference in Ca^{2+} signaling in the nervous system is a decreased capacity of aged neurons to maintain a steady resting state following a stimulation-evoked Ca^{2+} response. The calcium hypothesis of neuronal aging postulates that age-dependent dysregulation of calcium homeostasis that result in changes in the free intracellular Ca^{2+} concentration account for changes in neuronal function (Biessels and Gispen, 1996). The mechanisms underlying age-dependent dysregulations of Ca^{2+} homeostasis have been explored in detail in both the PNS and CNS (Pottorf et al., 2002; Toescu and Verkhratsky, 2003, 2004; Toescu et al., 2004). Several mechanisms contribute to this age-related decrease in Ca^{2+} homeostasis, such as increased Ca^{2+} entry due to increased VGCC expression, decreased Ca^{2+} uptake by ER and mitochondria, and/or decreased activity of plasma membrane ATP-dependent Ca^{2+} pumps (Kirischuk et al., 1992; Hartmann et al., 1996; Kirischuk and Verkhratsky, 1996; Xiong et al., 2002; Murchison et al., 2004; Vanterpool et al., 2005). The most common and constant observation is decreased mitochondrial Ca^{2+} uptake due to alteration of mitochondria function with aging. Ca^{2+} uptake by mitochondria not only determines Ca^{2+} signals but also regulates neuronal excitability (Nowicky and Duchen, 1998; Vanden Berghe et al., 2002). Studies have shown a critical role of mitochondria in intracellular Ca^{2+} signaling in DRG neurons (Dedov and Roufogalis, 2000; Dedov et al., 2001; Shishkin et al., 2002), suggesting that age-induced functional damages to mitochondria might have a significant effect on sensory function.

Although the mechanisms underlying age-related changes in intracellular Ca^{2+} signaling have been well investigated, less is known about membrane Ca^{2+}-permeable channels that trigger the Ca^{2+} signals (Griffith et al., 2000; Toescu and Verkhratsky, 2000). In this study we were particularly interested in the Ca^{2+}-permeable TRPV1 channel. To investigate whether functional properties of TRPV1 in primary sensory neurons change with aging, we compared the Ca^{2+} transients induced by application of 1uM capsaicin (CAP), a highly specific TRPV1 agonist, to DRG neurons from 2~4 month old and 15~18 month old male mice. We found that CAP-induced Ca^{2+} transients were slower and longer in aged DRG neurons. We also tested whether addition of ART to growth media altered TRPV1 activation in neurons of aged mice. Our findings show that ART has less of an effect on TRPV1 activity in aged neurons compared to young neurons, suggesting that aging does fundamentally alter the physiological activity of TRPV1.

6.2 CAP-INDUCED RESPONSES DEPEND ON MEMBRANE TRPV1

Capsaicin is a specific agonist of TRPV1 and increases intracellular Ca^{2+} by binding and opening plasma membrane TRPV1, which results in extracellular Ca^{2+} influx through this receptor. It may also act directly on ER and mitochondria and thereby induce Ca^{2+} release from internal stores. We first tested if application of 1uM CAP for 5s to DRG neurons had a specific effect on plasma membrane receptors but no other side effects. We found that brief application of a small amount of CAP to both young and old neurons induced very small responses in the absence of extracellular Ca^{2+} but robust responses in the presence of Ca^{2+} (**Figure 3.1A**), indicating that activation of plasma membrane TRPV1 by CAP triggered Ca^{2+} transients that were followed by a cascade of Ca^{2+}-buffering mechanisms. We then went on to compare amplitude (ΔF), latency (T_{max}) and half-decay time ($T_{1/2}$) of CAP-induced Ca^{2+} transients between young and old neurons to determine if functional properties of TRPV1 significantly change with aging. These data will be used in design of future detailed electrophysiological studies of aging neurons.

6.3 EFFECTS OF ARTEMIN ON CAP RESPONSES IN DRG NEURONS

To determine how ART regulates TRPV1 function in DRG neurons of Harlan NIA Blk6 mice, we first compared CAP responses in young neurons pooled from all levels of DRG cultured overnight (18~24h) in media containing 250ng/ml ART to neurons in media containing no growth factor or 50ng/ml NGF. CAP responsive neurons [CAP(+)] are defined as cells with one change in amplitude (ΔF) of Ca^{2+} transients greater than 0.2 following three separate CAP applications (**Figure 3.1B**). Compared with control neurons (CON) grown without any growth factors, neither ART nor NGF increased the percentage of CAP(+) neurons (CON, 32%; ART, 29%; NGF, 33%) among the total neurons (CON, $n = 164$; ART, $n = 166$; NGF, $n = 167$) investigated and responsive to 50mM KCl (**Table 6.1**). However, among all the CAP(+) cells (CON, $n = 52$; ART, $n = 48$; NGF, $n = 55$), ~20% in CON and NGF (CON, $n = 11$; NGF, $n = 11$) did not respond to the first CAP exposure (CAP1), whereas only ~10% in ART ($n = 5$) were not responsive to CAP1, i.e., ~90% of ART-treated CAP(+) cells ($n = 43$) were CAP1 responsive [CAP1(+)] (**Table 6.1**). The high percentage of CAP1 responders in ART media indicates that ART may enhance TRPV1 activation by the first CAP application.

Table 6.1 Effect of ART and NGF on percentage of CAP (+) neurons in young mice. DRG neurons isolated from young animals were incubated in media containing ART (ART) or NGF (NGF) or in media without growth factors (CON) overnight. The following day neurons were briefly exposed to 1uM CAP every ten minutes for three times (Figure 3.1B). CAP (+) (CAP-responsive) neurons are neurons responsive to either one of the three CAP applications. CAP1 (+) neurons are neurons that can respond to the first CAP exposure.

	Total neurons (*n*)	CAP(+) neurons (*n*)	% of CAP(+) in total neurons	CAP1(+) neurons (*n*)	% of CAP1(+) in CAP(+) neurons
CON	164	52	32%	41	79%
ART	166	48	29%	43	90%
NGF	167	55	33%	44	80%
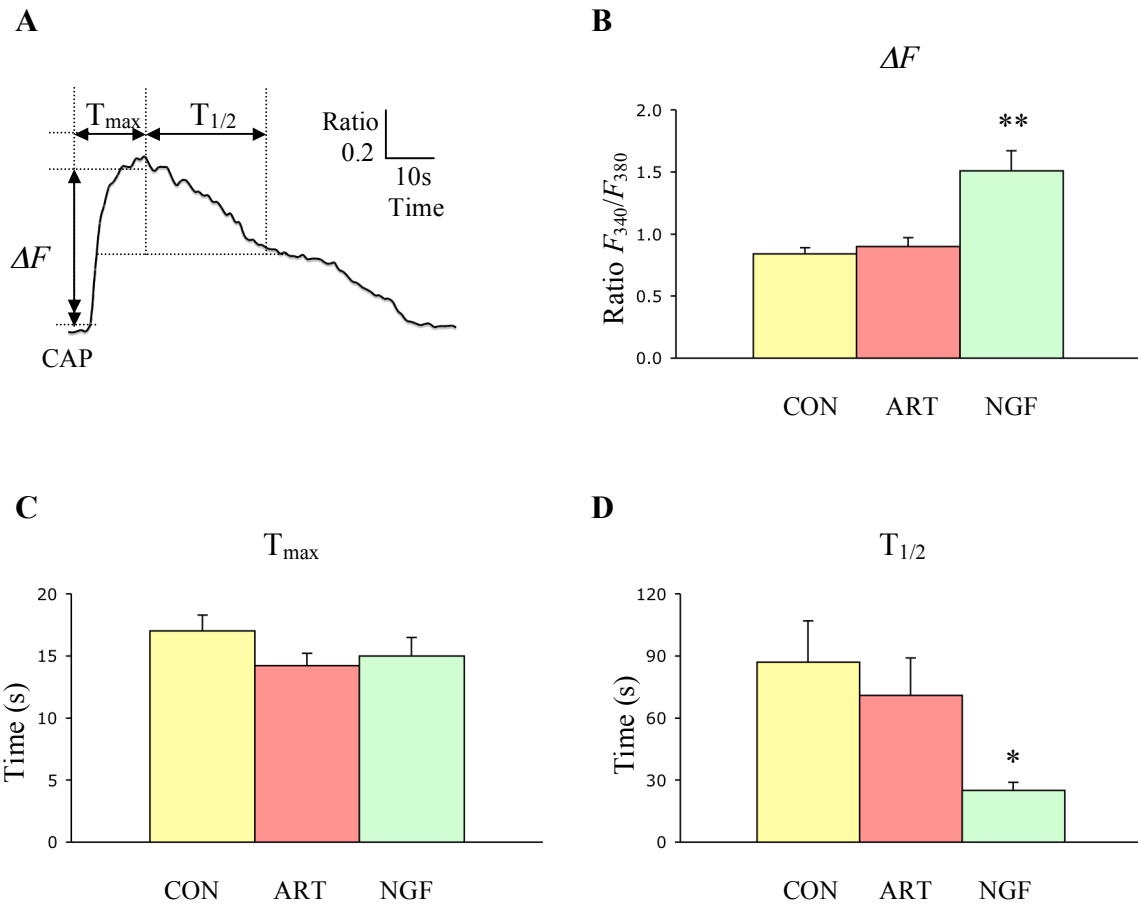
Figure 6.1 Effect of ART and NGF on CAP1 responses in young neurons. **A.** Parameters of Ca^{2+} transients induced by CAP (see **section 3.4.4** for detailed explanation). **B.** NGF in media increased the mean amplitude (ΔF) of Ca^{2+} transients but ART did not. **C.** The presence of ART or NGF in media did not affect the mean latency to maximal responses (T_{max}). **D.** NGF in media decreased the mean half-decay time ($T_{1/2}$) of Ca^{2+} transients but ART did not relative to control value. CON, $n = 32$; ART, $n = 24$; NGF, $n = 32$; * - $P < 0.05$, ** - $P < 0.01$

Next we looked at whether ART or NGF affected CAP1-induced Ca^{2+} transients in young neurons (**Figure 6.1**). Surprisingly ART ($n = 24$) had no effect on ΔF (0.9 ± 0.07) or $T_{1/2}$ ($71 \pm 18s$) while NGF ($n = 32$) significantly increased ΔF (1.51 ± 0.16, $P < 0.01$) and decreased $T_{1/2}$ ($25 \pm 4s$, $P < 0.05$) of CAP1-induced Ca^{2+} transients compared with control neurons (ΔF, 0.84 ± 0.05; $T_{1/2}$, $87 \pm 20s$; $n = 32$) (**Figure 6.1B** and **D**). Neither ART nor NGF affected T_{max} (CON, $17 \pm 1.3s$; ART, $14.2 \pm 1.1s$; NGF, $15 \pm 1.5s$) (**Figure 6.1C**). These findings indicate that long-term (18~24h) treatment with ART does not change physiological properties of TRPV1-dependent Ca^{2+} signals in CAP1-responsive DRG neurons, but NGF alone can enhance CAP1 responses.

6.4 EFFECTS OF ART ON CAP RESPONSES IN AGED NEURONS

To determine if ART or NGF modulate TRPV1 responses differentially in DRG neurons from young and old mice, we compared CAP responses in young and old neurons incubated in growth media containing both 250ng/ml ART and 50ng/ml NGF with those in neurons cultured in media containing 50ng/ml NGF alone. For studies of old neurons, cultures grown without growth factors could not be used for controls since old neurons do not survive in media without NGF. Thus for these studies, all neurons (young and old) were plated with 50ng/ml NGF. ART plus NGF (A+N) significantly increased the percentage of CAP-responsive cells in both young (45%, $P < 0.01$) and old animals (42%, $P < 0.01$) compared with NGF alone (young, 33%; old, 30%) (**Table 6.2**). But no difference in the percentage of CAP (+) neurons was detected between young and old animals, which is consistent with the immunolabeling study showing no difference in the percent of TRPV1 neurons in young and old ganglia (Figure 4.3B).

As shown in Table 6.1, ART alone may increase the percentage of CAP1-responsive cells in cultures of young neurons. In addition, exposure of young neurons to ART plus NGF significantly increased the percentage of CAP1 (+) neurons (94%, $P < 0.05$) compared with NGF alone (80%). In contrast to these findings, no significant increase in CAP1 responsive neurons was found in cultures from aged mice (A+N, 87%; NGF, 81%) (**Table 6.2**). Taken together,

Table 6.2 Effect of ART on CAP (+) neurons in young and old mice. The protocol used is similar to the one described in Table 6.1 except that DRG neurons from young and old animals were incubated in media containing NGF or NGF plus ART overnight.

	Total neurons (n)	CAP(+) neurons (n)	% of CAP(+) in total neurons	CAP1(+) neurons (n)	% of CAP1(+) in CAP(+) neurons
Y(N)	167	55	33%	44	80%
Y(NA)	117	53	45% **	50	94% *
O(N)	210	62	30%	50	80%
O(NA)	197	83	42% **	72	87%

Note: Y(N) or Y(NA) - young neurons grown in media containing NGF or NGF plus ART

O(N) or O(NA) – old neurons grown in media containing NGF or NGF plus ART

* - $P < 0.05$, ** - $P < 0.01$, χ^2 test, compared NGF plus ART to NGF group of the same age.

these studies indicate that ART has less effect on TRPV1 activation in old neurons, and this reduction may be related to the decreased level of GFRα3 in ganglia of aged animals.

To determine whether aging affects TRPV1 responsiveness, we compared CAP1-induced Ca^{2+} transients in young ($n = 32$) and old ($n = 34$) neurons cultured in media with NGF alone. Though there was no difference in the amplitude between young and old cells (young, 1.51 ± 0.16; old, 1.31 ± 0.1) (**Figure 6.2B**), it took old neurons about twice as long to reach the peak responsiveness (T_{max}, young, 14.7 ± 1.5s; old, 26.1 ± 3.2s; $P < 0.01$) (**Figure 6.2A** and **C**) as well as to recover ($T_{1/2}$, young, 25 ± 4s; old, 58 ± 12s; $P < 0.05$) (**Figure 6.2A** and **D**). This suggests that CAP-induced activity of plasma membrane TRPV1 is functionally changed with aging. To investigate ART effects on age-related changes in CAP-induced Ca^{2+} transients, we further analyzed young ($n = 30$) and old CAP1 (+) neurons ($n = 38$) cultured in media containing both ART and NGF. Similar to NGF alone, young neurons reached the maximal response in half the time taken by old neurons (T_{max}, young, 15 ± 1.1s; old, 31 ± 3.9s; $P < 0.01$) (**Figure 6.2C**). However, in the presence of ART and NGF the mean ΔF was significantly lower in young
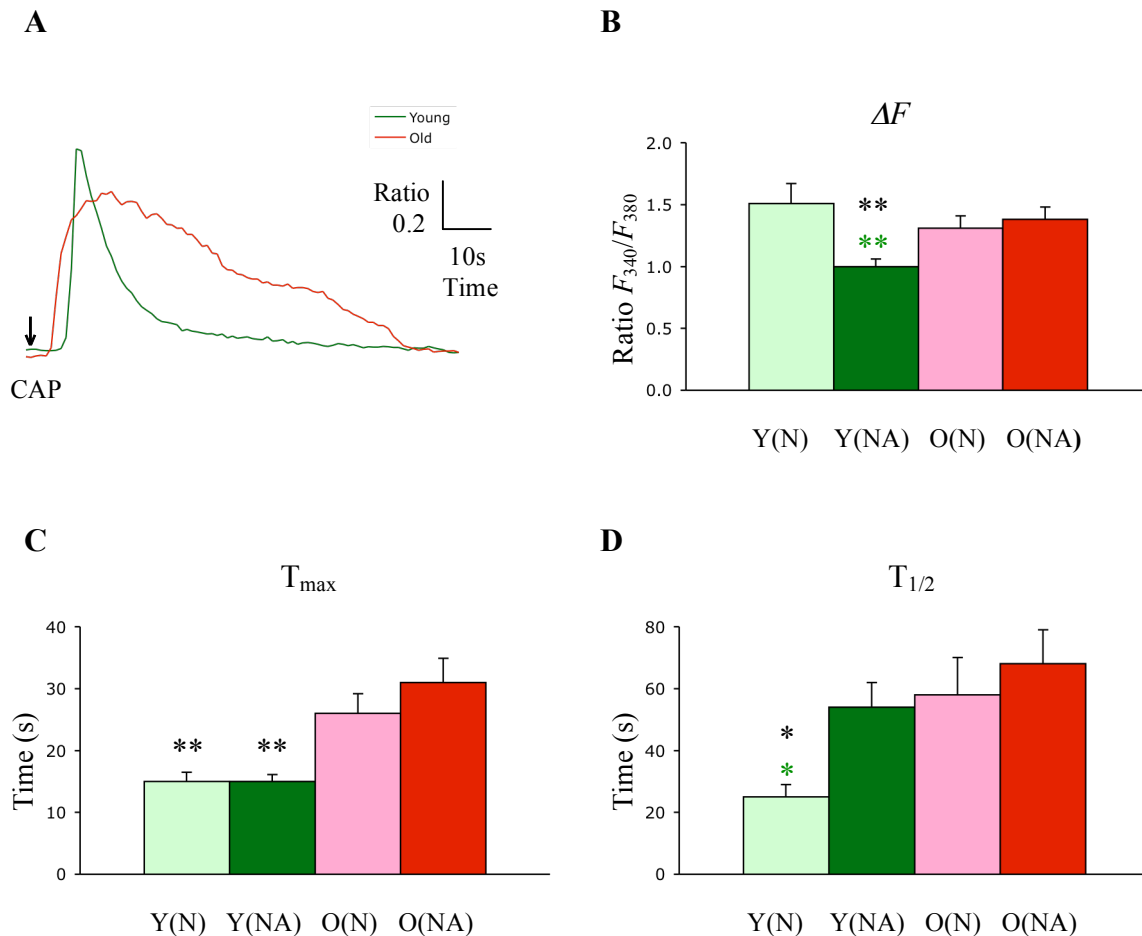
Figure 6.2 Effect of ART on CAP1 responses in young and old neurons. The same protocol was used as the one described in Figure 6.3. **A.** Comparison of a typical CAP1-induced Ca^{2+} transient in young and old neurons. **B.** ART and NGF in media decreased the mean ΔF of Ca^{2+} transients in young neurons compared to young in NGF (green asterisks) or old neurons (black asterisks). **C.** The mean latency to maximal responses (T_{max}) in young neurons was shorter than in old neurons (black asterisks), which was not affected by ART. **D.** The mean half-decay time ($T_{1/2}$) of Ca^{2+} transients in young neurons in NGF alone was shorter than in old neurons (black asterisk). ART in media increased $T_{1/2}$ in young neurons (green asterisk) but not in old neurons. Y(N) ($n = 32$) or Y(NA) ($n = 30$) - young neurons grown in media containing NGF or NGF plus ART, O(N) ($n = 34$) or O(NA) ($n = 38$) – old neurons grown in media containing NGF or NGF plus ART,* - $P < 0.05$, ** - $P < 0.01$

69

neurons (young, 1.0 ± 0.06; old, 1.38 ± 0.1; $P < 0.01$) (**Figure 6.2B**) while no difference was found in recovery between the two age groups (**Figure 6.2D**). ART plus NGF treatment also decreased the amplitude and increased the recovery time in young neurons compared with NGF treatment alone, but no changes were found in old neurons with or without ART (**Figure 6.2B** and **D**). These results suggest that ART has less of an effect on TRPV1 activation by CAP in DRG neurons isolated from aged mice.

6.5 EFFECTS OF AGING ON KCL-INDUCED RESPONSES

The relationship between calcium influx via membrane VGCCs and neuronal function in aging has been well studied in the nervous system. Consistent findings in cultured aged neurons include increased calcium influx via high threshold VGCCs, delayed recovery of intracellular Ca^{2+} and decreased neuronal excitability. To determine whether ART has effects on age-related changes in VGCC-dependent Ca^{2+} signals, we first analyzed KCl-induced Ca^{2+} transients in CAP1-sensitive young ($n = 18$) and old ($n = 20$) neurons incubated in NGF-containing media. Longer recovery was found in KCl-induced Ca^{2+} transients in old neurons ($T_{1/2}$, young, $10.1 \pm 3.7s$; old, $52.1 \pm 15.5s$; $P < 0.05$) (**Figure 6.3B**), indicating that functional properties of VGCCs may be altered with aging, which is consistent with previous studies. There was no change in amplitude between young and old cells (young, 1.36 ± 0.12; old, 1.36 ± 0.11) (**Figure 6.3A**). However, in the presence of both ART and NGF in the growth media, the mean amplitude of KCl-induced Ca^{2+} transients in 21 young neurons analyzed was significantly lower than in 23 old neurons (ΔF, young, 1.03 ± 0.05; old, 1.37 ± 0.12; $P < 0.05$). No difference was found in $T_{1/2}$ between young and old neurons (young, $19 \pm 4s$; old, $21 \pm 5s$) (**Figure 6.3A** and **B**), indicating that ART also has an effect on VGCC-dependent Ca^{2+} transients. Further analysis showed that in young neurons ART plus NGF treatment also decreased the amplitude of Ca^{2+} transients compared to NGF treatment alone ($P < 0.05$) whereas no difference was found in old neurons (**Figure 6.3A**). More interestingly, ART plus NGF significantly decreased $T_{1/2}$ in old neurons ($P < 0.05$) but had no effect on young neurons (**Figure 6.3B**). These findings show that age-related changes in DRG neurons alter the effect of ART on VGCC activation and/or expression.

A

ΔF

B

$T_{1/2}$
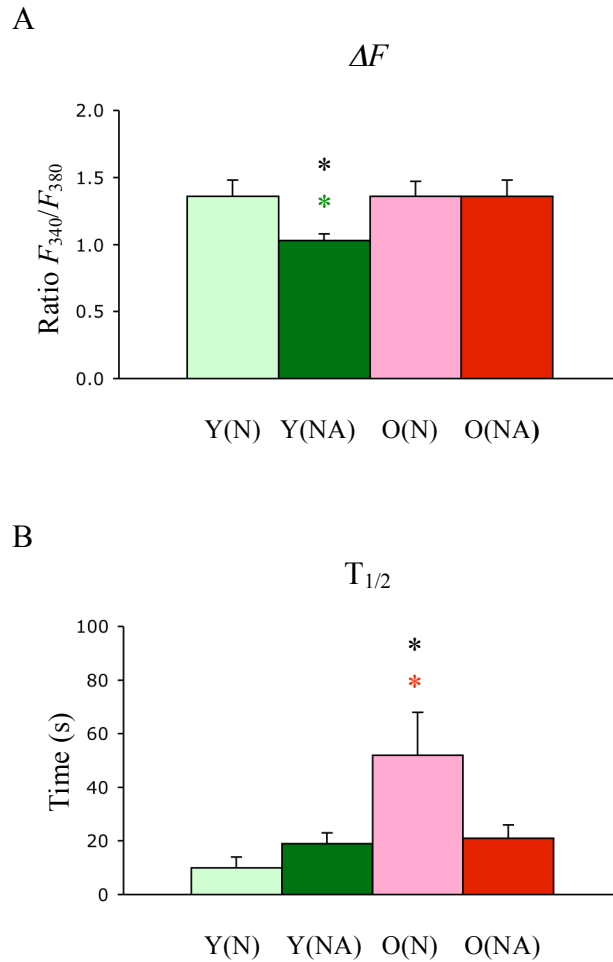
Figure 6.3 KCl-induced Ca^{2+} transients in CAP1 (+) young and old neurons. **A.** ART decreased the mean ΔF of Ca^{2+} transients in young neurons relative to those found in young neurons grown in NGF (green asterisks) or old neurons (black asterisks). **B.** The mean half-decay time ($T_{1/2}$) of Ca^{2+} transients in old neurons was longer than in young neurons (black asterisk). ART decreased $T_{1/2}$ in old neurons (red asterisk) but not in young neurons. Y(N) ($n = 18$) or Y(NA) ($n = 21$) - young neurons grown in media with NGF or NGF plus ART, O(N) ($n = 20$) or O(NA) ($n = 23$) - old neurons grown in media with NGF or NGF plus ART, * - $P < 0.05$

71

6.6 EFFECTS OF ARTEMIN ON TRPV1 FUNCTIONAL PROPERTIES

TRPV1 can be desensitized by repetitive application of capsaicin. ART, NGF as well as the nucleotide ATP can reverse repetitive CAP-induced desensitization and increase TRPV1 activation (Bonnington and McNaughton, 2003; Moriyama et al., 2003; Malin et al., 2006). Desensitization of TRPV1 following its activation and potentiation by ATP is critical in blocking or promoting pain transmission, especially during inflammation. To test if ART alone can modulate these functional properties of TRPV1, we measured TRPV1 desensitization by repetitive application of CAP and potentiation by ATP in young neurons grown overnight in media either containing no growth factor or ART alone (**Figure 6.4**). Without growth factors the CAP2 responses in young DRG neurons ($n = 37$) showed typical desensitization with 39% decrease in the mean response amplitude compared to CAP1 responses ($P < 0.01$) (**Figure 6.4A** and **B**). Potentiation of the CAP3 responses by ATP compared to CAP2 responses also occurred ($P < 0.05$) with 30% increase in response amplitude in cultures without growth factors (**Figure 6.4C** and **D**). In the presence of ART ($n = 40$) reduced desensitization occurred to only 19%, whereas potentiation by ATP occurred to only 12%, and neither desensitization nor potentiation was significant (**Figure 6.4B** and **D**). These changes indicate that ART alone in the media can inhibit TRPV1 desensitization as well as its potentiation by ATP.

To determine if age modulates desensitization and potentiation of CAP responses by ATP, young ($n = 39$) and old neurons ($n = 50$) cultured in NGF-containing media were compared (**Figure 6.5**). TRPV1 was desensitized following CAP activation by 19% in young neurons and 20% in old neurons. In NGF-only cultures ATP potentiation was increased 25% in young neurons and 32% in old neurons. Thus, young and old neurons showed comparable TRPV1 desensitization and potentiation in the presence of NGF. To test if ART induces age-related differences in these properties of TRPV1, young ($n = 39$) and old neurons ($n = 43$) cultured in media containing both ART and NGF were compared (**Figure 6.5**). ART plus NGF treatment did not significantly affect the desensitization shown by NGF alone, since a 17% decrease in young neurons and a 26% decrease in old neurons occurred. It did, however, dramatically decrease TRPV1 potentiation by ATP, where only a 4% and a 9% increase were measured in young and old neurons, respectively (**Figure 6.5B**). Similar to NGF alone, NGF and ART affected TRPV1
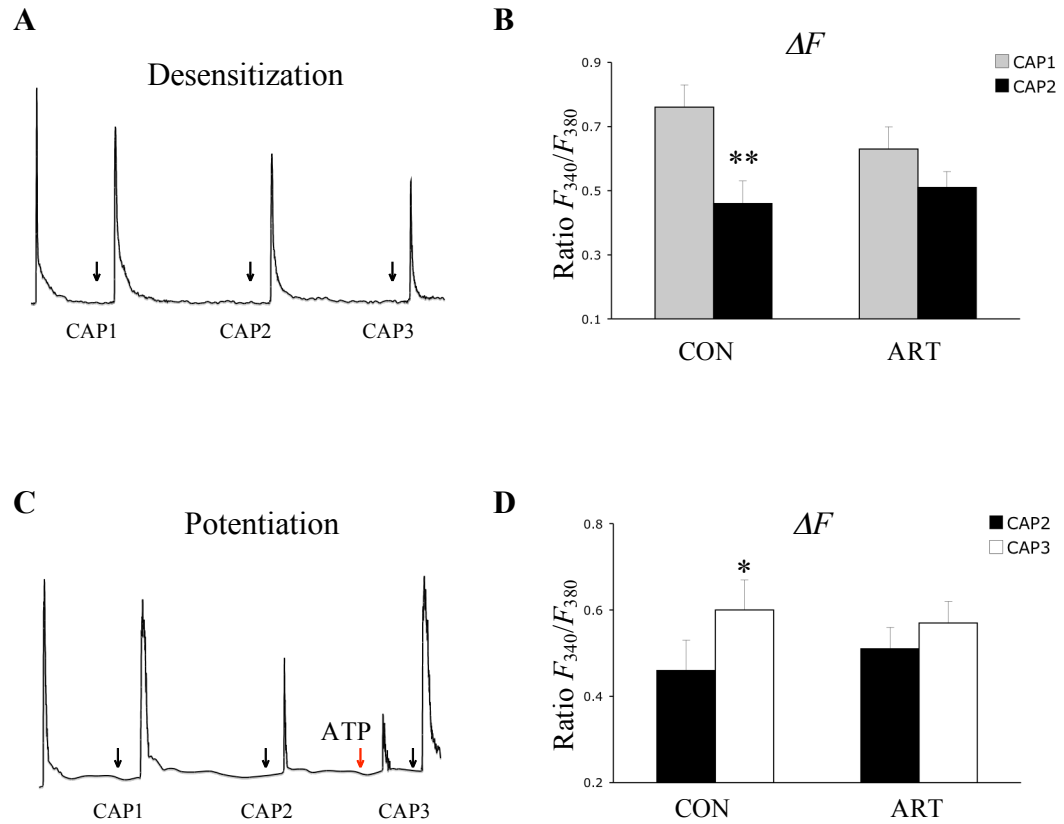
Figure 6.4 Effect of ART on TRPV1 desensitization and potentiation. Young DRG neurons were incubated in media containing ART (ART, $n = 40$) or in media without growth factors (CON, $n = 37$) overnight. **A.** Repetitive application of CAP decreases CAP response indicating TRPV1 is desensitized. **B.** In CON the CAP2 response is significantly reduced compared to CAP1, whereas with ART in media no significant difference is measured between CAP1 and CAP2 responses . **C.** Following ATP application (red arrow), CAP3 response is increased indicating TRPV1 is potentiated. **D.** In CON the CAP3 response is significantly increased compared to CAP2, while with ART in media no significant increase in CAP3 response is detected compared to CAP2. * - $P < 0.05$, ** - $P < 0.01$, paired t-test.

A

Desensitization

B
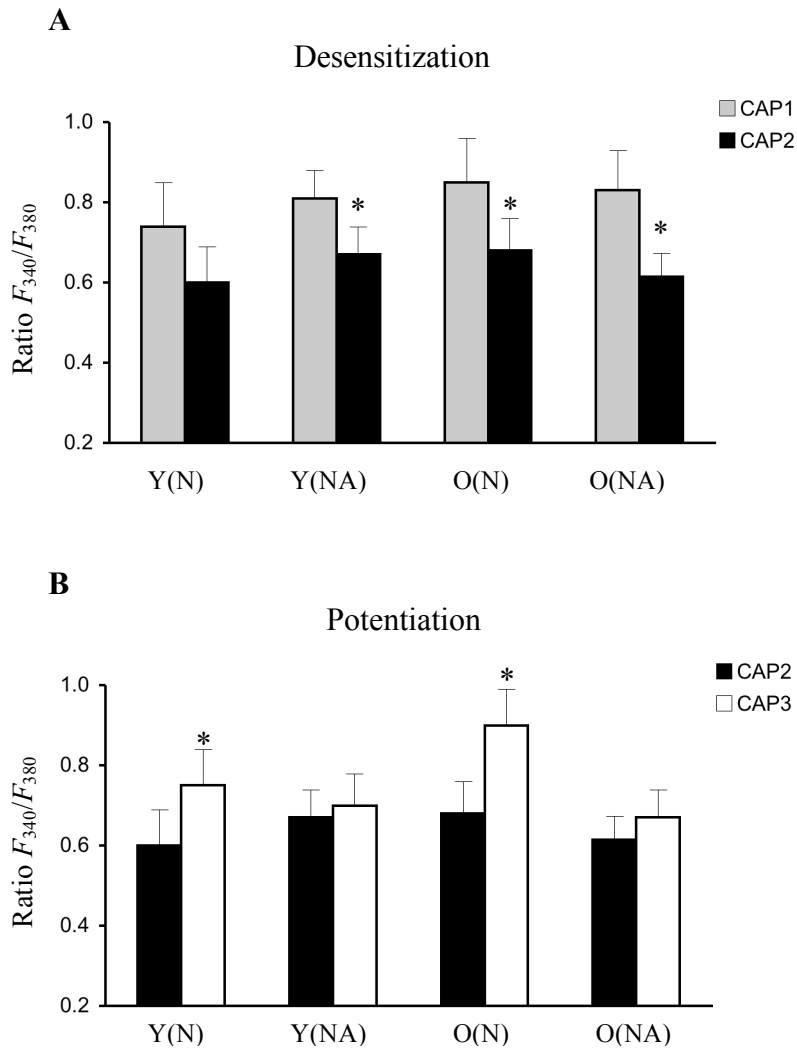
Potentiation

Figure 6.5 TRPV1 desensitization and potentiation in young and old neurons incubated in media containing NGF or NGF plus ART overnight. **A.** TRPV1 desensitization. With ART in media both young and old neurons show TRPV1 desensitization manifested by significantly reduced CAP2 responses compared to CAP1. **B.** TRPV1 potentiation by ATP. Age did not affect CAP3 potentiation by ATP though ART inhibited CAP3 potentiation in both young and old neurons. Y(N) (n = 39) or Y(NA) (n = 39) – young neurons grown in media containing NGF or NGF plus ART, O(N) (n = 50) or O(NA) (n = 43) – old neurons grown in media containing NGF or NGF plus ART, * - $P < 0.05$, paired t-test

response in young and old neurons to equal degree. Thus, age does not modulate TRPV1 desensitization and potentiation. It should be mentioned that not all of the analyzed neurons showed potentiation or desensitization of TRPV1 and no difference in the percentage of these neurons was found with aging. We also found that acute exposure to ART potentiated CAP responses in both young and old neurons growing in NGF-containing media. More studies are required to determine if there are age differences in the extent of potentiation and the percentage of potentiated neurons under these acute conditions.

6.7 CONCLUSIONS

To determine whether ART regulated TRPV1 function, CAP responses in young neurons grown overnight in media containing no growth factor, ART or NGF alone were compared. We found that NGF alone did increase CAP1 responses in young neurons compared to neurons grown without NGF, suggesting NGF enhances TRPV1 function *in vitro*. In contrast to the effect of NGF, ART alone rarely increased CAP1 responses and also decreased TRPV1 potentiation by ATP, indicating that overnight culture in ART may inhibit TRPV1 function *in vitro*. To determine whether age modulates the effect of ART on TRPV1 function, young and old neurons grown in media containing ART plus NGF or NGF alone were compared. We found that ART plus NGF, compared with NGF alone, decreased CAP1 responses in young neurons and inhibited TRPV1 potentiation by ATP in both young and old neurons, further supporting the inhibitory effects of ART on TRPV1 activity. These findings indicate that chronic exposure to ART may have an analgesic effect on TRPV1-mediated sensation. Decreased GFRα3 protein found in aged DRG may contribute to the age-modulated effects of ART on TRPV1 function and expression.

VII. DISCUSSION AND CONCLUSIONS

Somatosensory function has been shown to generally decrease with aging. Experiments of this dissertation investigated possible mechanisms that may underlie this age-related decline in sensory perception. Our studies reveal a complex interaction between growth factor and receptor expression, the level of ion channel expression and activity and expression of inflammatory proteins. The data suggest that a decline in expression of the artemin receptor GFRα3 in sensory ganglia occurs with aging and may underlie at least some aspects of the changes in sensory function. Similar to what has been shown for NGF, we propose that the growth factor artemin has two roles: it functions as a survival and differentiation factor during embryonic and postnatal development and as a modulator of afferent sensitivity in the adult. It is postulated that artemin signaling through the GFRα3 receptor declines in aging neurons, and this decline leads to down-regulation of TRPV1 translation and functional deficits in TRPV1 activation, which leads to impaired nociceptive signaling. Our findings also suggest that the "inflammatory tone" of an animal may contribute to pain threshold. Thus, the reduced pain sensitivity exhibited by the Harlan Blk6 mice relative to Blk6 mice from Jackson labs following CFA injection, may relate to the difference in expression of inflammatory mediators such as IL-6 and LIF. In addition, the increased expression of inflammation and immune related genes in ganglia of aged mice, determined using Affymetrix analysis, may contribute to the reduced sensitivity of older mice.

7.1 MOLECULAR BASIS FOR THERMAL SENSITIVITY IN AGING

7.1.1 TRPV1 expression and thermal sensitivity in aging

In our behavioral studies we show that a decrease in thermosensation occurs in naïve mice that are either 16 mo or 2 yr-old. Prior studies of age-associated thermal insensitivity in rodents have focused on the rat (Gagliese and Melzack, 2000). Outcomes of these studies are quite varied, with some reporting reduced thresholds in aged rats and others showing no change. These different outcomes could reflect the rat strain tested or variability in the test used (tail flick and hot plate) to measure thermal responsiveness. In this study we took great care to minimize possible environmental variables, i.e., animals were acclimated to the testing environment, they were tested at the same time of day by the same investigators and we used a radiant heat source applied to a discrete area of the foot with responses timed within 0.1 s (i.e., the Hargreaves' test). As in all behavioral studies of aged animals, a potential caveat is whether motor abilities are impaired in older animals that could slow an avoidance reflex. Although this remains a possibility, the 16-month and 2-year old animals used in this study showed no discernable impairment in mobility or motor control.

To determine whether the reduced detection of noxious heat in aging mice correlates with changes in ion channel expression in DRG and axons that project to the skin, we measured the relative level of TRPV1 in young and old mice. TRPV1, a heat-sensitive channel, is required for detection of heat at high temperatures ($\geq$ 52°C) *in vivo* and essential for thermal hyperalgesia induced by inflammation (Caterina et al., 2000; Davis et al., 2000; Ji et al., 2002). Our findings support a mechanism whereby a reduced level of TRPV1 contributes to a decrease in thermal sensitivity observed in both naïve and inflamed aging mice. This reduction in TRPV1 appeared to be more prominent in neurons at lumbar levels that project to the limbs, supporting the notion that long axonal length is a hindrance to transport of substances important for normal neuron function in aged animals (Caterina et al., 1997).

Under normal condition both 16-month and 2-year old mice exhibited reduced levels of TRPV1 protein in DRG neurons, though no change in the overall number of TRPV1-positive

neurons occurred. This suggests that changes in protein expression that contribute to sensory perception begin at midlife. This decrease in TRPV1 was greater in the 2-year old group indicating a progressive decline. Reduced levels of TRPV1 were also found in peripheral nerves of aged animals suggesting TRPV1 transport is also less efficient. Coincident with these processes, it is also possible that some TRPV1-positive fibers are lost with age due to neuronal death or degeneration of afferents in the periphery. Indeed, the lower density of immunolabeled TRPV1-positive fibers in tibial and cutaneous nerves would support this possibility. However, anatomical studies have indicated that loss of afferents in the skin becomes most prominent only in very old animals (Ceballos et al., 1999). Only a modest afferent loss would therefore be expected at 15 months of age, the time at which a reduction in TRPV1 protein was measured in this study. Consistent with the findings under normal conditions, impaired thermal hyperalgesia and lower level of TRPV1 proteins in peripheral nerves were also detected in aged mice following CFA-induced inflammation, supporting the requirement of TRPV1 for thermal sensitivity following inflammation. Further analysis indicates that the decreased TRPV1 protein might be related to an increased level of inflammation. This possibility is supported by the CFA-induced reduction of TRPV1 in the PNS of both young and aged mice. Interestingly, our results show that the reduction in TRPV1 in the DRG occurs only at the translational level. This mode of TRPV1 regulation is not unique since translational regulation of TRPV1 expression has also been reported in rat following inflammation of the footpad (Ji et al., 2002).

7.1.2 Functional properties of TRPV1 in DRG neurons in aging

Based on our behavioral and molecular findings in the PNS of aged mice, we hypothesized that age-related functional changes in TRPV1 might also occur. To test this hypothesis we examined TRPV1 activity in dissociated DRG neurons. We used Ca^{2+} imaging in order to monitor a large number of individual cells simultaneously. Physiological properties of TRPV1, a Ca^{2+}-permeable channel, were determined by analyzing TRPV1-dependent Ca^{2+} transients induced by application of capsaicin, a specific agonist of TRPV1. A study of rat DRG neurons has shown that about 97% of CAP-responsive neurons are also sensitive to heat although only 86% of heat-sensitive neurons respond to CAP (Savidge et al., 2001). So activation of TRPV1 by CAP reflects heat-activated TRPV1 function to a large degree although the molecular mechanisms of CAP

activation may be different from heat activation. Consistent with the finding that age does not affect the percent of TRPV1 neurons in DRG, we did not find a difference in the percentage of CAP–responsive cells between young and old animals *in vitro*. However, the percentage of CAP (+) cells (~30%) detected *in vitro*, which is similar to the percent of CAP-responsive neurons found in mouse trigeminal ganglia (Simonetti et al., 2006), is less than the percent of immunolabeled TRPV1-positive neurons (~50%) found in our *in vivo* studies and other *in vitro* studies (Simonetti et al., 2006), suggesting that some TRPV1 positive neurons may be functionally insensitive to CAP application.

Despite the fact that the percent of capsaicin-responsive neurons did not change with aging, analysis of Ca^{2+} transients did reveal changes in that the rising and recovery of CAP- induced Ca^{2+} transients in old neurons were significantly slower than in young neurons. The longer latency in reaching maximal response in old neurons was correlated with, and might also account for, the behavioral outcome where old animals took longer to withdraw from noxious heat. Because CAP is lipophilic and membrane permeable, and can pass through the cell membrane and act on binding sites in the cytosolic domain of TRPV1 (Tominaga and Tominaga, 2005), such an apparent time lag between CAP uptake and peak response in aging neurons might be explained in part by changes in membrane properties of old neurons. As mentioned before, neurons are enriched in unsaturated lipids, which are prone to oxidative damage, and accumulation of lipid damage can decrease fluidity and increase rigidity of the plasma membrane. These changes may lead to a decline in membrane receptor-mediated signaling and in turn a decrease in function (Shigenaga et al., 1994). The extended recovery of Ca^{2+} transients exhibited by old neurons may also reflect well-established age-related changes in cellular properties. In this case, slow recovery of Ca^{2+} level may be due to age-induced mitochondrial dysfunction, which causes intracellular Ca^{2+} dysregulation (Toescu, 2000; Toescu et al., 2000).

Although it is possible that age-related membrane and mitochondrial damage contribute to the slow and long Ca^{2+} transients induced by CAP in old neurons, we cannot rule out the possibility that the properties of TRPV1 receptor activation and inactivation also change with aging. Two types of CAP-induced inward currents: one with rapid activation (RA) and rapid inactivation (RI) and one with slow activation (SA) and slow inactivation (SI), have been

described in rat trigeminal ganglia. Both currents are about the same magnitude and CAP-responsive neurons have been shown to contain either one or both of the two types of currents (Marsh et al., 1987; Liu and Simon, 1994). Our results suggest that SA-SI currents may be more predominant in old neurons while RA-RI currents predominate in young neurons.

Phosphorylation of TRPV1 on certain amino acid residues is required for CAP binding, providing a way in which to control activation of ligand-gated channels (Jung et al., 2004). Selective phosphorylation of TRPV1 might explain why only some immuno-labeled TRPV1-positive neurons were activated by CAP. Slow activation of TRPV1 by CAP binding in old neurons might arise from reduced kinase phosphorylation or increased phosphatase action on TRPV1. Mechanisms underlying inactivation of TRPV1 vary with different kinetic components. Rapid inactivation depends on Ca^{2+} influx through TRPV1 while slow inactivation does not (Tominaga and Tominaga, 2005). Some studies have found that Ca^{2+}-dependent phosphorylation of TRPV1 regulates rapid inactivation while PKA-dependent phosphorylation of TRPV1 mediates slow inactivation (Docherty et al., 1996; Bhave et al., 2002). Thus, phosphorylation of TRPV1 by different mechanism seems to control TRPV1 activity through the dynamic balance between phosphorylation and dephosphorylation (Mohapatra and Nau, 2005; Jeske et al., 2006), which might be significantly altered during age. Further electrophysiological and phosphorylation assay studies are required to test this possibility.

7.1.3 Contribution of other factors to reduced thermal sensitivity in aging

7.1.3.1 Contribution of ion channels other than TRPV1. Although our studies suggest that down-regulation of TRPV1 expression and function in the PNS with age may contribute to decreased thermal sensitivity, especially decreased inflammatory thermal hyperalgesia, it should be mentioned that TRPV1 knockout mice and isolated nociceptors from them have normal heat responses (Caterina et al., 2000; Davis et al., 2000; Woodbury et al., 2004). Therefore TRPV1 is not the only channel involved in thermal nociception and a decrease in TRPV1 alone may not be enough to cause impaired thermal sensitivity in aged mice. However, in addition to decreased TRPV1 expression, the relative levels of mRNAs encoding the Nav1.8 and Nav1.9 sodium channels and Cav2.2 calcium channel were also reduced in DRG of old mice. These channels are

preferentially expressed by nociceptors, many of which are TRPV1 positive and responsive to heat. In rat DRG about 66% of TRPV1 neurons express Nav1.8 and 55% of TRPV1 cells co-express Nav1.9 (Amaya et al., 2000). *In vitro* studies show that 80% of CAP-responsive neurons express Nav1.8 mRNA, and a splicing variant of Cav2.2, e37a mRNA, is present in 55% of CAP-responsive neurons while only 17% of non-responsive cells are positive (Bell et al., 2004). Although the Nav1.8, Nav1.9 and Cav2.2 channels are not directly activated by heat, they are essential for generation and propagation of the action potential and neurotransmission in the nociceptive pathway that follows a heat stimulus. Nav1.8 channels, in particular, contribute more than 50% of the inward current underlying the depolarizing phase of the action potential in cells in which they are present, and endow cells with the capability to generate sustained trains of action potentials in response to long-lasting stimuli (Renganathan et al., 2001). Thus, the reduction in Nav1.8, Nav1.9 and Cav2.2 levels in the DRG, though modest, could impair nerve function. Likewise, modest reduction of TRPV channel proteins in the DRG cell bodies and afferents could alter the heat threshold of firing.

7.1.3.2 Contribution of glial and supporting cells. Supporting glial cells such as satellite cells and Schwann cells are also important for maintenance of normal neuronal function. For example, selective disruption of the ErbB4 receptor in adult non-myelinating Schwann cells causes loss of unmyelinated nerve fibers and impaired heat sensitivity (Chen et al., 2003). ETRB, an endothelin receptor, is specifically expressed in DRG satellite cells and nonmyelinating ensheathing Schwann cells and is also involved in signaling nociceptive events in peripheral tissues (Pomonis et al., 2001). A decreased level of ETRB and myelin mRNAs were expressed in DRG from aging mice, indicating that aging also compromises glial function which may modify pain sensitivity.

7.2 ARTEMIN-GFRα3 SIGNALING IN AGING AND INFLAMMATION

Trophic factor reduction in aging has been hypothesized to influence neuronal phenotype, e.g., by altering channel and peptide expression and thereby causing loss of functional sensitivity. Trophic signaling may decline due to structural changes in axons that impair retrograde (and

anterograde) transport of growth factors and their receptors. In the ganglia, paracrine and autocrine trophic signaling may also be affected. Indeed, decreases in Trk tyrosine kinase receptors (which bind the neurotrophins NGF, NT3, NT4 and BDNF) have been reported in sensory neurons of the 30-month old rat, supporting the notion that trophic signaling declines with age (Bergman et al., 1999a). Although an age-related reduction may not be sufficient to cause neuronal death, it may compromise regulation of gene expression. To understand the molecular mechanisms underlying down-regulation of TRPV1 expression and function in the PNS by aging, we investigated whether age modulated NGF, ART and receptor expression in the PNS. In mouse DRG about 67% of TRPV1 neurons express the ART receptor GFRα3, 80% of which are also NGF receptor TrkA-positive, suggesting that NGF and ART play a major role in regulation of TRPV1 expression and function. With this in mind, we examined trophic support in the aged ganglia by measuring the relative expression of TrkA and GFRα3 receptors. Results show the relative abundance of GFRα3 is decreased in aged ganglia on both transcriptional and translational levels in lumbar DRG while no change was detected in TrkA mRNA, suggesting GFRα3-dependent signaling but not TrkA, is selectively affected in aging systems. Though a decrease was found in GFRα3, our RT-PCR measures showed an increase in RET receptor expression. This increase could reflect an attempt to compensate for the decrease in GFRα3, which may occur in response to decreased production of trophic factors or decreased retrograde transport due to degenerative changes in peripheral afferents. However, both NGF and ART mRNA expression increased in the aging ganglia while no significant loss of target-derived neurotrophic factors was detected. Immunocytochemistry showed that the increase in ART expression occurred in satellite cells of the ganglia. Whether this increase is related to the reduction in GFRa3 is unclear (see below).

NGF and ART are involved in thermal hyperalgesia induced by inflammation, and NGF and ART expression has been reported to increase in inflamed tissue. Surprisingly, in our studies CFA injection resulted in a decrease in NGF and an increase in ART mRNA in inflamed skin on day 1. This increase in ART was substantially less (2.5-fold vs. 10-fold) compared to other studies using young male mice from Jackson labs on the same genetic background, which may explain shorter duration of hypersensitivity exhibited by the Harlan mice. We also found that increased ART in inflamed skin correlated with a decrease in GFRα3 expression in both ganglia

and peripheral nerves, and that as ART increased, GFRα3 level decreased. This suggests that increasing the concentration of ART in peripheral tissues can down-regulate GFRα3 expression. Although the mechanisms underlying regulation of GFRα3 expression by ART are unknown, production of ART in satellite cells of the ganglia may have a role. Results show an increase in ART protein in satellite cells of the aging ganglia. Ganglia and nerves of aging mice were found to have less GFRα3 protein. This reduction in GFRα3 may therefore result from feedback caused by the increase in ART found in supporting satellite cells.

Sensory ganglia from aging animals were also found to have an up-regulation of genes associated with immune/inflammation responses. In DRG and peripheral nerves multiple types of cells have immune-like function, including Schwann cells, satellite cells, endothelial cells, dendritic cells and macrophages etc., and therefore could be producing these transcripts. Immune activation of these cells could potentially release inflammatory cytokines as well as growth factors like NGF and thereby induce an inflammatory response (Watkins and Maier, 2002). Inflammatory cytokines can interact with growth factors as well as regulate expression of growth factors, which is especially true for NGF during inflammation. LIF has been shown to inhibit NGF expression in skin and diminish thermal hyperalgesia induced by CFA (Banner et al., 1998). In our CFA study we found that IL-6 had a linear inhibitory effect on ART expression in skin following inflammation, suggesting that ART might also contribute to inflammation, which can be regulated by other inflammatory cytokines. It has been reported that CFA-induced up-regulation of IL-6 in inflamed tissue is dramatically inhibited in LIF knockout mice compare to wild-type animals (Zhu et al., 2001). In the comparative study of Harlan and Jackson mice, the different fold changes of IL-6 in inflamed skin might be due therefore, to the difference in baseline level of LIF in the Harlan and Jackson mouse skin. Similarly, the lower baseline level of LIF in skin of aged animals could explain, in part, their reduced sensitivity following CFA injection. In conjunction with the findings of increased ART and decreased GFRα3 in aged ganglia and following inflammation, we think it possible that ART may act as an inflammatory mediator involved in chronic inflammation and as such, contribute to age-induced changes in the peripheral nociceptive system.

7.3 REGULATION OF NOCICEPTOR FUNCTION BY ART IN AGING

7.3.1 Regulation of TRPV1 expression by ART

Regulation of TRPV1 expression and function by NGF has been well studied using *in vivo* and *in vitro* model systems. These studies have shown that NGF not only up-regulates TRPV1 expression but also sensitizes TRPV1 activity via TrkA-mediated signaling pathways in nociceptors, and contributes to inflammation-induced thermal hyperalgesia (Ji et al., 2002; Bonnington and McNaughton, 2003; Zhang et al., 2005). Our results using calcium imaging are consistent with these findings. We found that NGF up-regulates TRPV1 expression as reflected by greater CAP responses in young DRG neurons cultured in NGF-containing media compared to neurons in media without growth factor (Ji et al., 2002; Zhang et al., 2005). Moreover, in the presence of NGF alone in the growth media, no difference in either the percentage of CAP (+) neurons or the magnitude of CAP responses between young and old neurons was found, consistent with our assays showing TrkA mRNA level in aged ganglia was similar to that in young ganglia. Our findings also support the idea that TrkA-mediated neurotrophic signaling is not changed in DRG with age, which has been demonstrated in other studies (Jiang and Smith, 1995; Jiang et al., 1995; Hall et al., 2001). More recent studies have shown that ART also regulates TRPV1 expression and function and when injected causes acute thermal hypersensitivity. Indeed the regulatory effects of ART on TRPV1 are even greater than NGF (Elitt et al., 2006; Malin et al., 2006). However, comparison of young DRG neurons grown overnight in ART-containing media with those cultured in media without growth factor showed no significant difference in the percent of CAP (+) neurons or the magnitude of CAP responses. This suggests that ART alone may not be sufficient to regulate TRPV1 expression in sensory neurons. Another possibility however, is that overnight exposure of cultured neurons to high levels of ART desensitized TRPV1, reducing the CAP response.

As mentioned above, previous studies have shown that NGF alone can increase TRPV1 expression via TrkA signaling and this effect is not affected by aging. Interestingly, we found that adding ART into NGF-containing media caused a reduction in the magnitude of CAP responses in young DRG neurons but no change in old neurons, indicating that ART may down-

regulate TRPV1 protein when added with NGF. If so, this effect of ART may depend entirely on GFRα3 signaling, which is decreased in aged ganglia. This could explain why old neurons are less sensitive to ART treatment. Although the detailed mechanisms of TRPV1 regulation by ART are not clear, GFRα3 is the only known receptor to date that can specifically bind to ART and is thought to be required for ART effects on TRPV1. This is supported by the finding that up-regulation of GFRα3 in DRG of mice with ART overexpression and during inflammation is correlated with increased expression of TRPV1 in the PNS and thermal hypersensitivity (Elitt et al., 2006; Malin et al., 2006). We also found that aging and inflammation-induced down-regulation of GFRα3 was correlated with reduced TRPV1 expression in the PNS and impaired thermal sensitivity in aged mice, and further confirmed involvement of GFRα3 in regulation of TRPV1-dependent nociception. However, in our studies we did not find a positive correlation between ART and GFRα3 in the PNS as reported in other studies (Ceyhan et al., 2006; Elitt et al., 2006). Thus, how ART regulates GFRα3-dependent signaling remains unclear.

7.3.2 Regulation of TRPV1 function by ART

Although addition of ART decreased the magnitude of CAP responses in young neurons grown in media with NGF, the percentage of CAP (+) neurons cultured in NGF and ART-containing media was increased from ~30% to ~40% in both young and old animals. Since the amplitude of CAP responses was decreased in young neurons, the greater percentage of CAP (+) neurons could not be due to increased expression of TRPV1. It is very possible that TRPV1 function or activity is upregulated by ART in the presence of NGF. ART alone can potentiate CAP responses and reverse TRPV1 desensitization by repetitive application of CAP *in vitro*, which has been confirmed by our own studies. Phosphorylation is one important mechanism underlying sensitization of TRPV1 by NGF, ATP and other inflammatory mediators (Tominaga and Tominaga, 2005), and maybe also by ART via GFRα3-RET signaling. As discussed earlier, phosphorylation is required for activation of TRPV1 by CAP binding, and CAP cannot activate less phosphorylated or dephosphorylated TRPV1. ART alone in the media increased the percentage of CAP1 (+) neurons that can be activated by the first exposure to CAP, indicating that TRPV1 is more easily activated by CAP in the presence of ART in spite of no significant effect on TRPV1 expression or function found in young neurons, and this effect may be due to

increased phosphorylation by ART. In the presence of ART with NGF in media more TRPV1 might be phosphorylated and then easily activated by CAP, though the magnitude of CAP responses decrease. The overall result may be what we observed; that more neurons respond to CAP and among them more neurons are activated by the first CAP application. Since TRPV1 phosphorylation via ART may also require GFRα3, less GFRα3 found in aged ganglia may account for less increase in the percentage of CAP1 (+) neurons dissociated from aged ganglia following addition of ART into NGF-containing media. More interestingly, potentiation of TRPV1 by ATP was inhibited in young neurons cultured in media containing ART alone, and addition of ART into media containing NGF inhibited ATP potentiation in both young and old neurons. PKC-dependent phosphorylation of TRPV1 is involved in sensitization of TRPV1 by ATP, which can bind and activate GPCRs such as P2Y2 which in turn activate downstream PKC (Tominaga et al., 2001). Inhibition of ATP potentiation by ART suggest that ART and ATP may share the same downstream PKC-dependent pathways and/or the same phosphorylation sites in TRPV1 such as Ser 502 and Ser 800 (Numazaki et al., 2002).

7.3.3 Regulation of VGCC expression by ART

Previous studies have shown age-dependent alterations in VGCCs in both CNS and PNS, which can affect neuronal excitability and function (Kostyuk et al., 1993; Murchison and Griffith, 1995; Landfield, 1996). We also detected decreased gene expression of Cav2.2 in aged DRG. Since one splicing variant of Cav2.2, e37a, has been found preferentially expressed in CAP responsive DRG neurons, 67% of which also express GFRα3, it is possible that overexpression of ART may cause up-regulation of Cav2.2-e37a expression in sensory ganglia. Unexpectedly a 1.6-fold decrease of Cav2.2-e37a mRNAs was measured in lumbar DRG of ART-overexpressing mice (data not shown). Consistent with this finding, addition of ART into NGF-containing media decreased the amplitude of KCl-induced and VGCC-dependent Ca^{2+} transients in young CAP responsive neurons but not in old neurons which have less GFRα3 on the average, indicating that regulation of Cav2.2-e37a by ART can occur through GFRα3 signaling. Systemic treatment with ART has been found to relieve experimental neuropathic pain in rats by decreasing CAP-induced neurotransmitter release in the dorsal horn of the spinal cord (Gardell et al., 2003). A more recent study also found that ART could attenuate herpes-related pain responses in mice infected with

herpes simplex (Asano et al., 2006). Our results suggest that the analgesic effect of ART might rely on down-regulation of TRPV1 and Cav2.2 expression, and that this effect is absent in old neurons and therefore age-dependent. In contrast, the inhibitory effect of ART on TRPV1 potentiation by ATP is not affected by age. It should be noted that we used Ca^{2+} imaging to study TRPV1 expression and function *in vitro* by analyzing CAP induced TRPV1-dependent Ca^{2+} transients, which can reflect molecular and physiological properties of TRPV1 to some degree. It is not clear how well CAP induced Ca^{2+} transients correlate with TRPV1 expression and activity. However, most studies indicate that upregulation of TRPV1 increases CAP responses (Bonnington and McNaughton, 2003; Elitt et al., 2006; Malin et al., 2006).

7.3.4 Specificity of ART effect on nociceptors in aging

In mouse DRG the majority of TRPV1-positive neurons are NGF- responsive nociceptors, ~67% of which express GFRα3. Nearly all GFRα3-positive sensory neurons express TRPV1, suggesting that ART may differentially regulate NGF-dependent but not GDNF-dependent nociceptors in mice. This specificity has been supported by development studies. During development ART selectively supports a subpopulation of nociceptors expressing GFRα3 and the majority of them are NGF-responsive nociceptors. Our findings suggest that ART may also be specifically involved in aging of NGF-dependent nociceptors by regulation of GFRα3 signaling in mouse. ART and NGF are two major factors that regulate TRPV1-expressing NGF-responsive nociceptors whereas GDNF has less effect on TRPV1-mediated nociception. During aging GDNF-GFRα1 neurotrophic signaling does not change in DRG, therefore GDNF-dependent nociceptors should be less affected by age. Indeed, no age-related changes in gene expression or protein level of P2X3, a Ca^{2+}-permeable channel that can be activated by ATP and also predominantly expressed in GDNF-dependent nociceptors, were found in sensory ganglia, peripheral nerves and hind paw skin (data not shown). *In vitro* Ca^{2+} imaging studies, though lacking in specificity, also consistently showed that ATP induced Ca^{2+} transients in sensory neurons did not change with aging (data not shown). In contrast, GFRα3 signaling is decreased in aged DRG, which is correlated with increased ART and decreased TRPV1 expression and function despite no change in TrkA signaling in aged DRG. These findings indicate a specific effect of ART on TRPV1-expressing NGF-responsive nociceptors in mice during aging.

7.4 SUMMARY AND CONCLUSIONS

To understand the molecular mechanisms in aging of the peripheral nociceptive system, we compared gene expression associated with nociception, neurotrophic signaling, and immune /inflammation in the cutaneous sensory system between young and old mice under normal conditions and following inflammation. We found that gene expression of channels and receptors involved in nociception, such as Nav1.8, Nav1.9, Cav2.2 and ETRB, decreased in aged DRG. The levels of TRPV1 and Nav1.8 protein were also reduced in DRG or peripheral nerves with aging. Following inflammation TRPV1 protein was decreased in peripheral nerves in both young and old mice and this reduction correlated with reduced thermal hyperalgesia. These findings support a mechanism whereby these molecular changes contribute to impaired thermal sensation during aging. To determine whether altered neurotrophic signaling is involved in age-modulated expression of these channels and receptors, particularly TRPV1 in this study, we measured NGF/TrkA, GDNF/GFRα1 and ART/GFRα3 transcripts in DRG and found a reduction in GFRα3 and no change in TrkA or GFRα1, though the ligands NGF and ART increased in aged DRG. Inflammation further increased ART expression in inflamed skin and decreased GFRα3 expression in DRG or peripheral nerves in both young and old mice. Increased ART expression during inflammation is linearly correlated with expression of the inflammatory cytokine IL-6 in inflamed skin, suggesting that ART might also act as an inflammatory factor and interact with other inflammatory cytokines. The decrease in GFRα3 may result from an inflammation-induced increase in ART.

Aged animals exhibited an increase in genes associated with immune/inflammation, such as IL-6, and decreased expression of genes associated with glial support function, like ETRB and myelin, in the DRG. These findings suggest that age compromises the normal support function of glial cells and activates immune cells and/or immune-like glial cells in sensory ganglia. These activated immune or immune-like cells may contribute to increased expression of inflammatory genes and growth factors including ART and NGF in aged ganglia. Increased production of ART may selectively decrease GFRα3 signaling in nociceptors, and in turn down-regulate TRPV1 expression and function and reduce thermal sensitivity during aging (**Figure 7.1**). Consistent with the *in vivo* findings, *in vitro* studies of isolated DRG neurons using calcium imaging
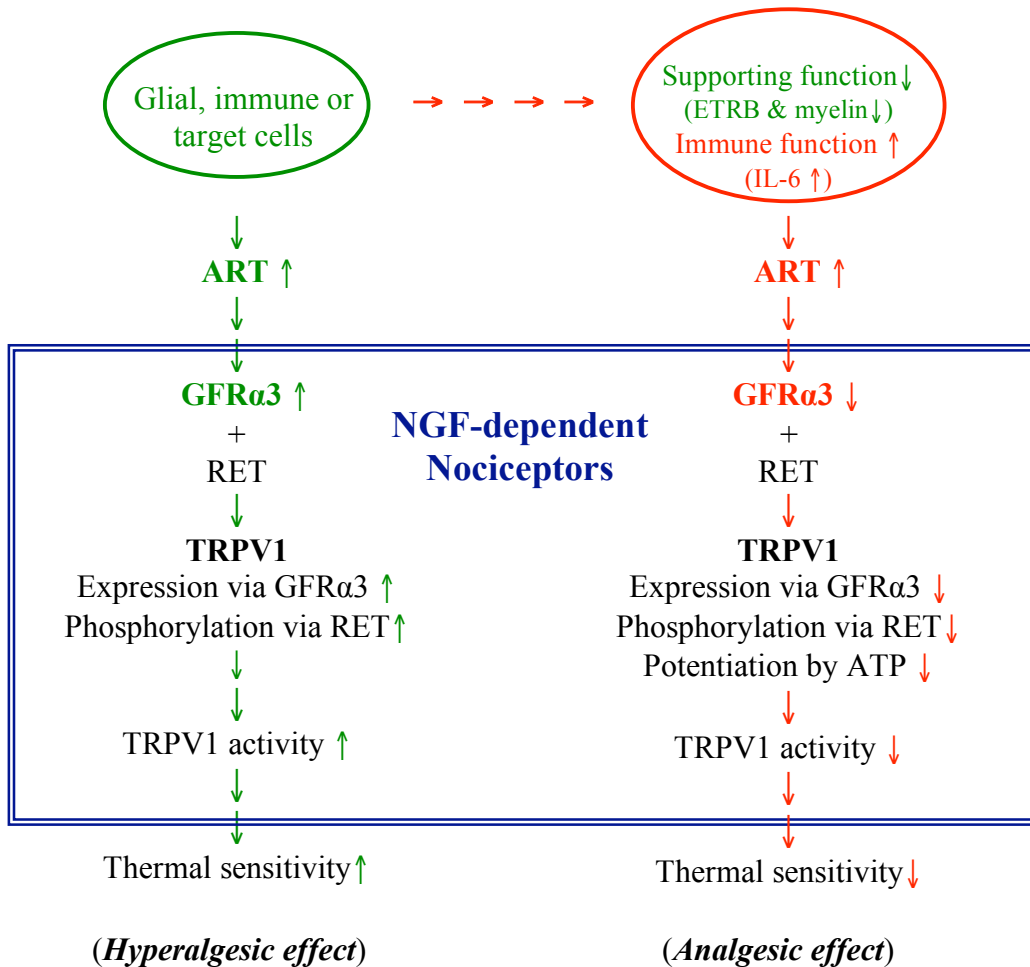
Figure 7.1 Paradoxical effects of ART on thermal nociception. During development and following acute inflammation, an increase in ART expression in glial, target and/or immune cells has a hyperalgesic effect on thermal sensation perhaps via up-regulation of GFRα3 and TRPV1 activity in NGF-dependent nociceptors. During aging and chronic inflammation, supporting function of glial, target and/or immune cells may be decreased while immune function is increased, and an increase in ART expression in these cells has an analgesic effect on thermal sensation probably via down-regulation of GFRα3 and TRPV1 activity in those nociceptors.

indicate that long-term exposure to ART decreases the amplitude of CAP-induced TRPV1-dependent Ca^{2+} transients. This effect is absent in aged neurons that have less GFRα3 found *in vivo*, suggesting that TRPV1 down-regulation by ART depends on GFRα3 signaling.

Together with other studies of ART in development and acute inflammation, two different and paradoxical effects of ART on thermal nociception have been found (**Figure 7.1**). During development and acute inflammation, an increase in ART expression has a hyperalgesic effect on nociceptors by upregulation of TRPV1 through GFRα3-RET signaling and induction of thermal hypersensitivity. During aging and chronic inflammation, increased ART expression has an analgesic effect on nociceptors by down-regulation of TRPV1 through the same GFRα3-RET signaling and reduce thermal sensitivity. In fact, the analgesic effect has already been shown by previous studies that ART treatment can relieve experimental pathological pain. Our studies also suggest that inflammation may be a major mechanism in aging of the peripheral nociceptive system, and an increase in ART expression and inflammatory related molecules by glial and immune-like cells in sensory ganglia may be a key factor in aging of NGF-dependent nociceptors in mice. Certainly, more evidence, such as aging of ART knockout mice, is required to support this theory.

Table 7.1 List of abbreviations

Abbreviation	Full name	Note
AP	Action potential	
AHP	Afterhyperpolarization	
ART	Artemin	Neurotrophic factor
ASIC	Acid-sensing ion channel	
BDNF	Brain-derived neurotrophic factor	
CAP	Capsaicin	TRPV1 agonist
Cav2.2	Voltage-gated calcium channel 2.2	
CFA	Complete Freund's adjuvant	Inflammatory reagent
CGRP	Calcitonin gene-related peptide	Neurotransmitter
CNS	Central nervous system	
DRG	Dorsal root ganglia	
ETRB	Endothelin receptor type B	
ER	Endoplasmic reticulum	
GDNF	Glial cell line-derived neurotrophic factor	
GFRα	GDNF family receptor alpha	
IL-6	Interleukin-6	Inflammatory cytokine
LIF	Leukemia inhibitory factor	Inflammatory cytokine
Nav1.8	Voltage-gated sodium channel 1.8	
NGF	Nerve growth factor	Neurotrophic factor
NTN	Neurturin	Neurotrophic factor
PNS	Peripheral nervous/nociceptive system	
TG	Trigeminal ganglia	
TRPV	Transient receptor potential vanilloid receptor	
VGCC	Voltage-gated calcium channel	

BIBLIOGRAPHY

Akopian AN, Souslova V, England S, Okuse K, Ogata N, Ure J, Smith A, Kerr BJ, McMahon SB, Boyce S, Hill R, Stanfa LC, Dickenson AH, Wood JN (1999) The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat Neurosci 2:541-548.

Albers KM, Woodbury CJ, Ritter AM, Davis BM, Koerber HR (2006) Glial cell-line-derived neurotrophic factor expression in skin alters the mechanical sensitivity of cutaneous nociceptors. J Neurosci 26:2981-2990.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J (1994) Molecular Biology of the Cell, 3rd Edition. New York: Graland Publishing.

Alvarez de la Rosa D, Zhang P, Shao D, White F, Canessa CM (2002) Functional implications of the localization and activity of acid-sensitive channels in rat peripheral nervous system. Proc Natl Acad Sci U S A 99:2326-2331.

Amaya F, Decosterd I, Samad TA, Plumpton C, Tate S, Mannion RJ, Costigan M, Woolf CJ (2000) Diversity of expression of the sensory neuron-specific TTX-resistant voltage-gated sodium ion channels SNS and SNS2. Mol Cell Neurosci 15:331-342.

Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, Suzuki H, Tanaka M (2004) NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. Eur J Neurosci 20:2303-2310.

Andrew D, Greenspan JD (1999) Peripheral coding of tonic mechanical cutaneous pain: comparison of nociceptor activity in rat and human psychophysics. J Neurophysiol 82:2641-2648.

Asano K, Asahina S, Sakai M, Matsuda T, Ou K, Maeda Y, Hisamitsu T (2006) Attenuating effect of artemin on herpes-related pain responses in mice infected with herpes simplex. In Vivo 20:533-537.

Banner LR, Patterson PH, Allchorne A, Poole S, Woolf CJ (1998) Leukemia inhibitory factor is an anti-inflammatory and analgesic cytokine. J Neurosci 18:5456-5462.

Barbacid M (1995) Neurotrophic factors and their receptors. Curr Opin Cell Biol 7:148-155.

Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Winter J, Bevan S, Wishart W, Fox A, Ganju P (2002) Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. J Neurosci 22:8139-8147.

Batchelor PE, Liberatore GT, Wong JY, Porritt MJ, Frerichs F, Donnan GA, Howells DW (1999) Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19:1708-1716.

Baudet C, Mikaels A, Westphal H, Johansen J, Johansen TE, Ernfors P (2000) Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins. Development 127:4335-4344.

Beitel RE, Dubner R, Harris R, Sumino R (1977) Role of thermoreceptive afferents in behavioral reaction times to warming temperature shifts applied to the monkeys face. Brain Res 138:329-346.

Bell TJ, Thaler C, Castiglioni AJ, Helton TD, Lipscombe D (2004) Cell-specific alternative splicing increases calcium channel current density in the pain pathway. Neuron 41:127-138.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet 38:515-517.

Bennett DL (2001) Neurotrophic factors: important regulators of nociceptive function. Neuroscientist 7:13-17.

Bennett DL, Boucher TJ, Armanini MP, Poulsen KT, Michael GJ, Priestley JV, Phillips HS, McMahon SB, Shelton DL (2000) The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. J Neurosci 20:427-437.

Bergman E, Ulfhake B (1998) Loss of primary sensory neurons in the very old rat: neuron number estimates using the disector method and confocal optical sectioning. J Comp Neurol 396:211-222.

Bergman E, Ulfhake B (2002) Evidence for loss of myelinated input to the spinal cord in senescent rats. Neurobiol Aging 23:271-286.

Bergman E, Fundin BT, Ulfhake B (1999a) Effects of aging and axotomy on the expression of neurotrophin receptors in primary sensory neurons. J Comp Neurol 410:368-386.

Bergman E, Kullberg S, Ming Y, Ulfhake B (1999b) Upregulation of GFRalpha-1 and c-ret in primary sensory neurons and spinal motoneurons of aged rats. J Neurosci Res 57:153-165.

Bergman E, Johnson H, Zhang X, Hokfelt T, Ulfhake B (1996) Neuropeptides and neurotrophin receptor mRNAs in primary sensory neurons of aged rats. J Comp Neurol 375:303-319.

Besne I, Descombes C, Breton L (2002) Effect of age and anatomical site on density of sensory innervation in human epidermis. Arch Dermatol 138:1445-1450.

Besson JM, Chaouch A (1987) Peripheral and spinal mechanisms of nociception. Physiol Rev 67:67-186.

Bevan S, Winter J (1995) Nerve growth factor (NGF) differentially regulates the chemosensitivity of adult rat cultured sensory neurons. J Neurosci 15:4918-4926.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 35:721-731.

Biessels G, Gispen WH (1996) The calcium hypothesis of brain aging and neurodegenerative disorders: significance in diabetic neuropathy. Life Sci 59:379-387.

Blair NT, Bean BP (2002) Roles of tetrodotoxin (TTX)-sensitive Na+ current, TTX-resistant Na+ current, and Ca2+ current in the action potentials of nociceptive sensory neurons. J Neurosci 22:10277-10290.

Blanchard BJ, Park T, Fripp WJ, Lerman LS, Ingram VM (1993) A mitochondrial DNA deletion in normally aging and in Alzheimer brain tissue. Neuroreport 4:799-802.

Bonnington JK, McNaughton PA (2003) Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. J Physiol 551:433-446.

Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB (2000) Potent analgesic effects of GDNF in neuropathic pain states. Science 290:124-127.

Cacalano G, Farinas I, Wang LC, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF, Hynes M, Davies A, Rosenthal A (1998) GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21:53-62.

Campbell LW, Hao SY, Thibault O, Blalock EM, Landfield PW (1996) Aging changes in voltage-gated calcium currents in hippocampal CA1 neurons. J Neurosci 16:6286-6295.

Cardenas CG, Del Mar LP, Scroggs RS (1995) Variation in serotonergic inhibition of calcium channel currents in four types of rat sensory neurons differentiated by membrane properties. J Neurophysiol 74:1870-1879.

Casolini P, Catalani A, Zuena AR, Angelucci L (2002) Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat. J Neurosci Res 68:337-343.

Caterina MJ, Julius D (1999) Sense and specificity: a molecular identity for nociceptors. Curr Opin Neurobiol 9:525-530.

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398:436-441.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306-313.

Ceballos D, Cuadras J, Verdu E, Navarro X (1999) Morphometric and ultrastructural changes with ageing in mouse peripheral nerve. J Anat 195 (Pt 4):563-576.

Ceyhan GO, Bergmann F, Kadihasanoglu M, Erkan M, Park W, Hinz U, Muller MW, Giese T, Buchler MW, Giese NA, Friess H (2006) The neurotrophic factor artemin influences the extent of neural damage and growth in chronic pancreatitis. Gut.

Chakour MC, Gibson SJ, Bradbeer M, Helme RD (1996) The effect of age on A delta- and C-fibre thermal pain perception. Pain 64:143-152.

Chan DC (2006) Mitochondria: dynamic organelles in disease, aging, and development. Cell 125:1241-1252.

Chao MV, Hempstead BL (1995) p75 and Trk: a two-receptor system. Trends Neurosci 18:321-326.

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377:428-431.

Chen S, Rio C, Ji RR, Dikkes P, Coggeshall RE, Woolf CJ, Corfas G (2003) Disruption of ErbB receptor signaling in adult non-myelinating Schwann cells causes progressive sensory loss. Nat Neurosci 6:1186-1193.

Chung HY, Kim HJ, Kim JW, Yu BP (2001) The inflammation hypothesis of aging: molecular modulation by calorie restriction. Ann N Y Acad Sci 928:327-335.

Chung HY, Sung B, Jung KJ, Zou Y, Yu BP (2006) The molecular inflammatory process in aging. Antioxid Redox Signal 8:572-581.

Cook SP, Vulchanova L, Hargreaves KM, Elde R, McCleskey EW (1997) Distinct ATP receptors on pain-sensing and stretch-sensing neurons. Nature 387:505-508.

Costigan M, Befort K, Karchewski L, Griffin RS, D'Urso D, Allchorne A, Sitarski J, Mannion JW, Pratt RE, Woolf CJ (2002) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci 3:16.

Cowen T (1993) Ageing in the autonomic nervous system: a result of nerve-target interactions? A review. Mech Ageing Dev 68:163-173.

Cowen T (2002) Selective vulnerability in adult and ageing mammalian neurons. Auton Neurosci 96:20-24.

Crisp T, Giles JR, Cruce WL, McBurney DL, Stuesse SL (2003) The effects of aging on thermal hyperalgesia and tactile-evoked allodynia using two models of peripheral mononeuropathy in the rat. Neurosci Lett 339:103-106.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J,

Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405:183-187.

Dedov VN, Roufogalis BD (2000) Mitochondrial calcium accumulation following activation of vanilloid (VR1) receptors by capsaicin in dorsal root ganglion neurons. Neuroscience 95:183-188.

Dedov VN, Mandadi S, Armati PJ, Verkhratsky A (2001) Capsaicin-induced depolarisation of mitochondria in dorsal root ganglion neurons is enhanced by vanilloid receptors. Neuroscience 103:219-226.

DeVane CL (2001) Substance P: a new era, a new role. Pharmacotherapy 21:1061-1069.

Disterhoft JF, Thompson LT, Moyer JR, Jr., Mogul DJ (1996) Calcium-dependent afterhyperpolarization and learning in young and aging hippocampus. Life Sci 59:413-420.

Djouhri L, Lawson SN (2004) Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Brain Res Rev 46:131-145.

Djouhri L, Bleazard L, Lawson SN (1998) Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J Physiol 513 (Pt 3):857-872.

Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN (2003) The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J Physiol 550:739-752.

Docherty RJ, Yeats JC, Bevan S, Boddeke HW (1996) Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. Pflugers Arch 431:828-837.

Dubner R, Bennett GJ (1983) Spinal and trigeminal mechanisms of nociception. Annu Rev Neurosci 6:381-418.

Elitt CM, McIlwrath SL, Lawson JJ, Malin SA, Molliver DC, Cornuet PK, Koerber HR, Davis BM, Albers KM (2006) Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. J Neurosci 26:8578-8587.

Erraji-Benchekroun L, Underwood MD, Arango V, Galfalvy H, Pavlidis P, Smyrniotopoulos P, Mann JJ, Sibille E (2005) Molecular aging in human prefrontal cortex is selective and continuous throughout adult life. Biol Psychiatry 57:549-558.

Etminan M, Gill S, Samii A (2003) Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. Bmj 327:128.

Evason K, Huang C, Yamben I, Covey DF, Kornfeld K (2005) Anticonvulsant medications extend worm life-span. Science 307:258-262.

Fang M, Wang Y, He QH, Sun YX, Deng LB, Wang XM, Han JS (2003) Glial cell line-derived neurotrophic factor contributes to delayed inflammatory hyperalgesia in adjuvant rat pain model. Neuroscience 117:503-512.

Fang X, Djouhri L, Black JA, Dib-Hajj SD, Waxman SG, Lawson SN (2002) The presence and role of the tetrodotoxin-resistant sodium channel Na(v)1.9 (NaN) in nociceptive primary afferent neurons. J Neurosci 22:7425-7433.

Fernandez HL, Hodges-Savola CA (1994) Axoplasmic transport of calcitonin gene-related peptide in rat peripheral nerve as a function of age. Neurochem Res 19:1369-1377.

Flanigan KM, Lauria G, Griffin JW, Kuncl RW (1998) Age-related biology and diseases of muscle and nerve. Neurol Clin 16:659-669.

Frank MG, Barrientos RM, Biedenkapp JC, Rudy JW, Watkins LR, Maier SF (2006) mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. Neurobiol Aging 27:717-722.

Gadient RA, Patterson PH (1999) Leukemia inhibitory factor, Interleukin 6, and other cytokines using the GP130 transducing receptor: roles in inflammation and injury. Stem Cells 17:127-137.

Gagliese L, Melzack R (1999) Age differences in the response to the formalin test in rats. Neurobiol Aging 20:699-707.

Gagliese L, Melzack R (2000) Age differences in nociception and pain behaviours in the rat. Neurosci Biobehav Rev 24:843-854.

Gardell LR, Wang R, Ehrenfels C, Ossipov MH, Rossomando AJ, Miller S, Buckley C, Cai AK, Tse A, Foley SF, Gong B, Walus L, Carmillo P, Worley D, Huang C, Engber T, Pepinsky B, Cate RL, Vanderah TW, Lai J, Sah DW, Porreca F (2003) Multiple actions of systemic artemin in experimental neuropathy. Nat Med 9:1383-1389.

Gavazzi I, Cowen T (1996) Can the neurotrophic hypothesis explain degeneration and loss of plasticity in mature and ageing autonomic nerves? J Auton Nerv Syst 58:1-10.

Gemma C, Mesches MH, Sepesi B, Choo K, Holmes DB, Bickford PC (2002) Diets enriched in foods with high antioxidant activity reverse age-induced decreases in cerebellar beta-adrenergic function and increases in proinflammatory cytokines. J Neurosci 22:6114-6120.

Gibson SJ, Helme RD (2001) Age-related differences in pain perception and report. Clin Geriatr Med 17:433-456, v-vi.

Gibson SJ, Farrell M (2004) A review of age differences in the neurophysiology of nociception and the perceptual experience of pain. Clin J Pain 20:227-239.

Gold MS (1999) Tetrodotoxin-resistant Na+ currents and inflammatory hyperalgesia. Proc Natl Acad Sci U S A 96:7645-7649.

Gold MS, Levine JD, Correa AM (1998) Modulation of TTX-R INa by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. J Neurosci 18:10345-10355.

Gooney M, Messaoudi E, Maher FO, Bramham CR, Lynch MA (2004) BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events. Neurobiol Aging 25:1323-1331.

Griffith WH, Jasek MC, Bain SH, Murchison D (2000) Modification of ion channels and calcium homeostasis of basal forebrain neurons during aging. Behav Brain Res 115:219-233.

Gruner W, Silva LR (1994) Omega-conotoxin sensitivity and presynaptic inhibition of glutamatergic sensory neurotransmission in vitro. J Neurosci 14:2800-2808.

Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. J Neurosci 22:6408-6414.

Guo A, Vulchanova L, Wang J, Li X, Elde R (1999) Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 11:946-958.

Hall KE, Sheng HC, Srinivasan S, Spitsbergen JM, Tuttle JB, Steers WD, Wiley JW (2001) Treatment of aged rat sensory neurons in short-term, serum-free culture with nerve growth factor reverses the effect of aging on neurite outgrowth, calcium currents, and neuronal survival. Brain Res 888:128-137.

Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A (2001) Does oxidative damage to DNA increase with age? Proc Natl Acad Sci U S A 98:10469-10474.

Harman D (2001) Aging: overview. Ann N Y Acad Sci 928:1-21.

Hartmann H, Eckert A, Velbinger K, Rewsin M, Muller WE (1996) Down-regulation of free intracellular calcium in dissociated brain cells of aged mice and rats. Life Sci 59:435-449.

Hattiangady B, Rao MS, Shetty GA, Shetty AK (2005) Brain-derived neurotrophic factor, phosphorylated cyclic AMP response element binding protein and neuropeptide Y decline as early as middle age in the dentate gyrus and CA1 and CA3 subfields of the hippocampus. Exp Neurol 195:353-371.

Heft MW, Cooper BY, O'Brien KK, Hemp E, O'Brien R (1996) Aging effects on the perception of noxious and non-noxious thermal stimuli applied to the face. Aging (Milano) 8:35-41.

Helme RD, Meliala A, Gibson SJ (2004) Methodologic factors which contribute to variations in experimental pain threshold reported for older people. Neurosci Lett 361:144-146.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EM, Jr., Milbrandt J (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. Neuron 22:253-263.

Honma Y, Araki T, Gianino S, Bruce A, Heuckeroth R, Johnson E, Milbrandt J (2002) Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. Neuron 35:267-282.

Hukkanen M, Platts LA, Corbett SA, Santavirta S, Polak JM, Konttinen YT (2002) Reciprocal age-related changes in GAP-43/B-50, substance P and calcitonin gene-related peptide (CGRP) expression in rat primary sensory neurones and their terminals in the dorsal horn of the spinal cord and subintima of the knee synovium. Neurosci Res 42:251-260.

Iggo A, Andres KH (1982) Morphology of cutaneous receptors. Annu Rev Neurosci 5:1-31.

Iwata K, Fukuoka T, Kondo E, Tsuboi Y, Tashiro A, Noguchi K, Masuda Y, Morimoto T, Kanda K (2002) Plastic changes in nociceptive transmission of the rat spinal cord with advancing age. J Neurophysiol 87:1086-1093.

Jeske NA, Patwardhan AM, Gamper N, Price TJ, Akopian AN, Hargreaves KM (2006) Cannabinoid WIN 55,212-2 regulates TRPV1 phosphorylation in sensory neurons. J Biol Chem 281:32879-32890.

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36:57-68.

Jiang ZG, Smith RA (1995) Regulation by nerve growth factor of neuropeptide phenotypes in primary cultured sensory neurons prepared from aged as well as adult mice. Brain Res Dev Brain Res 90:190-193.

Jiang ZG, Smith RA, Neilson MM (1995) The effects of nerve growth factor on neurite outgrowth from cultured adult and aged mouse sensory neurons. Brain Res Dev Brain Res 85:212-219.

Jourdan D, Boghossian S, Alloui A, Veyrat-Durebex C, Coudore MA, Eschalier A, Alliot J (2000) Age-related changes in nociception and effect of morphine in the Lou rat. Eur J Pain 4:291-300.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Julius D, McCleskey E (2006) Textbook of pain, 5th Edition. Churchill Livingston: Elsevier.

Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, Oh U (2004) Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. J Biol Chem 279:7048-7054.

Kenshalo DR, Sr. (1986) Somesthetic sensitivity in young and elderly humans. J Gerontol 41:732-742.

Kerr BJ, Souslova V, McMahon SB, Wood JN (2001) A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain. Neuroreport 12:3077-3080.

Kim DS, Yoon CH, Lee SJ, Park SY, Yoo HJ, Cho HJ (2001) Changes in voltage-gated calcium channel alpha(1) gene expression in rat dorsal root ganglia following peripheral nerve injury. Brain Res Mol Brain Res 96:151-156.

Kirischuk S, Verkhratsky A (1996) Calcium homeostasis in aged neurones. Life Sci 59:451-459.

Kirischuk S, Pronchuk N, Verkhratsky A (1992) Measurements of intracellular calcium in sensory neurons of adult and old rats. Neuroscience 50:947-951.

Kitagawa J, Kanda K, Sugiura M, Tsuboi Y, Ogawa A, Shimizu K, Koyama N, Kamo H, Watanabe T, Ren K, Iwata K (2005) Effect of chronic inflammation on dorsal horn nociceptive neurons in aged rats. J Neurophysiol 93:3594-3604.

Kodukula P, Liu T, Rooijen NV, Jager MJ, Hendricks RL (1999) Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. J Immunol 162:2895-2905.

Koltzenburg M, Stucky CL, Lewin GR (1997) Receptive properties of mouse sensory neurons innervating hairy skin. J Neurophysiol 78:1841-1850.

Koltzenburg M, Bennett DL, Shelton DL, McMahon SB (1999) Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. Eur J Neurosci 11:1698-1704.

Kostyuk P, Pronchuk N, Savchenko A, Verkhratsky A (1993) Calcium currents in aged rat dorsal root ganglion neurones. J Physiol 461:467-483.

Koyama Y, Tsujikawa K, Matsuda T, Baba A (2003a) Intracerebroventricular administration of an endothelin ETB receptor agonist increases expressions of GDNF and BDNF in rat brain. Eur J Neurosci 18:887-894.

Koyama Y, Tsujikawa K, Matsuda T, Baba A (2003b) Endothelin-1 stimulates glial cell line-derived neurotrophic factor expression in cultured rat astrocytes. Biochem Biophys Res Commun 303:1101-1105.

Krabbe KS, Pedersen M, Bruunsgaard H (2004) Inflammatory mediators in the elderly. Exp Gerontol 39:687-699.

Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, Khrapko K (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet 38:518-520.

Kregel KC, Zhang HJ (2006) An Integrated View of Oxidative Stress in Aging: Basic Mechanisms, Functional Effects and Pathological Considerations. Am J Physiol Regul Integr Comp Physiol.

La Forte RA, Melville S, Chung K, Coggeshall RE (1991) Absence of neurogenesis of adult rat dorsal root ganglion cells. Somatosens Mot Res 8:3-7.

Landfield PW (1996) Aging-related increase in hippocampal calcium channels. Life Sci 59:399-404.

Lapchak PA, Araujo DM, Beck KD, Finch CE, Johnson SA, Hefti F (1993) BDNF and trkB mRNA expression in the hippocampal formation of aging rats. Neurobiol Aging 14:121-126.

Lavrovsky Y, Chatterjee B, Clark RA, Roy AK (2000) Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. Exp Gerontol 35:521-532.

Lee CK, Weindruch R, Prolla TA (2000) Gene-expression profile of the ageing brain in mice. Nat Genet 25:294-297.

Lee CK, Klopp RG, Weindruch R, Prolla TA (1999) Gene expression profile of aging and its retardation by caloric restriction. Science 285:1390-1393.

Lewin GR, Ritter AM, Mendell LM (1993) Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci 13:2136-2148.

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432-435.

Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 103:525-535.

Lindfors PH, Voikar V, Rossi J, Airaksinen MS (2006) Deficient nonpeptidergic epidermis innervation and reduced inflammatory pain in glial cell line-derived neurotrophic factor family receptor alpha2 knock-out mice. J Neurosci 26:1953-1960.

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362-364.

Liu L, Simon SA (1994) A rapid capsaicin-activated current in rat trigeminal ganglion neurons. Proc Natl Acad Sci U S A 91:738-741.

Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429:883-891.

Macdonald NJ, Decorti F, Pappas TC, Taglialatela G (2000) Cytokine/neurotrophin interaction in the aged central nervous system. J Anat 197 Pt 4:543-551.

Magerl W, Fuchs PN, Meyer RA, Treede RD (2001) Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. Brain 124:1754-1764.

Malin SA, Molliver DC, Koerber HR, Cornuet P, Frye R, Albers KM, Davis BM (2006) Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. J Neurosci 26:8588-8599.

Mamet J, Baron A, Lazdunski M, Voilley N (2002) Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. J Neurosci 22:10662-10670.

Marsh SJ, Stansfeld CE, Brown DA, Davey R, McCarthy D (1987) The mechanism of action of capsaicin on sensory C-type neurons and their axons in vitro. Neuroscience 23:275-289.

Mattson MP, Magnus T (2006) Ageing and neuronal vulnerability. Nat Rev Neurosci 7:278-294.

McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW (1998) Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol 55:1513-1520.

McHugh JM, McHugh WB (2000) Pain: neuroanatomy, chemical mediators, and clinical implications. AACN Clin Issues 11:168-178.

McMahon SB, Bennett DL, Priestley JV, Shelton DL (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. Nat Med 1:774-780.

Mendell LM, Albers KM, Davis BM (1999) Neurotrophins, nociceptors, and pain. Microsc Res Tech 45:252-261.

Meyer RA, Ringkamp M, campbell JN, S.N R (2006) Textbook of pain, 5th Edition. Churhill Livingston: Elsevier.

Michael GJ, Priestley JV (1999) Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 19:1844-1854.

Millan MJ (1999) The induction of pain: an integrative review. Prog Neurobiol 57:1-164.

Ming Y, Bergman E, Edstrom E, Ulfhake B (1999a) Reciprocal changes in the expression of neurotrophin mRNAs in target tissues and peripheral nerves of aged rats. Neurosci Lett 273:187-190.

Ming Y, Bergman E, Edstrom E, Ulfhake B (1999b) Evidence for increased GDNF signaling in aged sensory and motor neurons. Neuroreport 10:1529-1535.

Mintz IM, Adams ME, Bean BP (1992) P-type calcium channels in rat central and peripheral neurons. Neuron 9:85-95.

Mohammed HA, Santer RM (2001) Total neuronal numbers of rat lumbosacral primary afferent neurons do not change with age. Neurosci Lett 304:149-152.

Mohapatra DP, Nau C (2005) Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. J Biol Chem 280:13424-13432.

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19:849-861.

Moore AR, Clinch D (2004) Underlying mechanisms of impaired visceral pain perception in older people. J Am Geriatr Soc 52:132-136.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382:76-79.

Moriyama T, Iida T, Kobayashi K, Higashi T, Fukuoka T, Tsumura H, Leon C, Suzuki N, Inoue K, Gachet C, Noguchi K, Tominaga M (2003) Possible involvement of P2Y2 metabotropic receptors in ATP-induced transient receptor potential vanilloid receptor 1-mediated thermal hypersensitivity. J Neurosci 23:6058-6062.

Murchison D, Griffith WH (1995) Low-voltage activated calcium currents increase in basal forebrain neurons from aged rats. J Neurophysiol 74:876-887.

Murchison D, Zawieja DC, Griffith WH (2004) Reduced mitochondrial buffering of voltage-gated calcium influx in aged rat basal forebrain neurons. Cell Calcium 36:61-75.

Nagley P, Zhang C, Lim ML, Merhi M, Needham BE, Khalil Z (2001) Mitochondrial DNA deletions parallel age-linked decline in rat sensory nerve function. Neurobiol Aging 22:635-643.

Nagy I, Rang H (1999) Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. Neuroscience 88:995-997.

Nickander KK, Schmelzer JD, Low PA (2002) Assessment of the "common" 4.8-kb mitochondrial DNA deletion and identification of several closely related deletions in the dorsal root ganglion of aging and streptozotocin rats. J Peripher Nerv Syst 7:96-103.

Nishino J, Mochida K, Ohfuji Y, Shimazaki T, Meno C, Ohishi S, Matsuda Y, Fujii H, Saijoh Y, Hamada H (1999) GFR alpha3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. Neuron 23:725-736.

North RA (2004) P2X3 receptors and peripheral pain mechanisms. J Physiol 554:301-308.

Nowicky AV, Duchen MR (1998) Changes in [Ca2+]i and membrane currents during impaired mitochondrial metabolism in dissociated rat hippocampal neurons. J Physiol 507 (Pt 1):131-145.

Numazaki M, Tominaga T, Toyooka H, Tominaga M (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. J Biol Chem 277:13375-13378.

Okuse K, Chaplan SR, McMahon SB, Luo ZD, Calcutt NA, Scott BP, Akopian AN, Wood JN (1997) Regulation of expression of the sensory neuron-specific sodium channel SNS in inflammatory and neuropathic pain. Mol Cell Neurosci 10:196-207.

Opree A, Kress M (2000) Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 20:6289-6293.

Orozco OE, Walus L, Sah DW, Pepinsky RB, Sanicola M (2001) GFRalpha3 is expressed predominantly in nociceptive sensory neurons. Eur J Neurosci 13:2177-2182.

Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A (2002) A heat-sensitive TRP channel expressed in keratinocytes. Science 296:2046-2049.

Peng YB, Ringkamp M, Meyer RA, Campbell JN (2003) Fatigue and paradoxical enhancement of heat response in C-fiber nociceptors from cross-modal excitation. J Neurosci 23:4766-4774.

Pomonis JD, Rogers SD, Peters CM, Ghilardi JR, Mantyh PW (2001) Expression and localization of endothelin receptors: implications for the involvement of peripheral glia in nociception. J Neurosci 21:999-1006.

Pottorf WJ, Duckles SP, Buchholz JN (2002) Aging and calcium buffering in adrenergic neurons. Auton Neurosci 96:2-7.

Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, Tarr DE, Sluka KA, Brennan TJ, Lewin GR, Welsh MJ (2001) The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 32:1071-1083.

Price MP, Lewin GR, McIlwrath SL, Cheng C, Xie J, Heppenstall PA, Stucky CL, Mannsfeldt AG, Brennan TJ, Drummond HA, Qiao J, Benson CJ, Tarr DE, Hrstka RF, Yang B, Williamson RA, Welsh MJ (2000) The mammalian sodium channel BNC1 is required for normal touch sensation. Nature 407:1007-1011.

Reichling DB, Levine JD (1999) The primary afferent nociceptor as pattern generator. Pain Suppl 6:S103-109.

Renganathan M, Cummins TR, Waxman SG (2001) Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. J Neurophysiol 86:629-640.

Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, Meyer RA (2001) Capsaicin responses in heat-sensitive and heat-insensitive A-fiber nociceptors. J Neurosci 21:4460-4468.

Rossi J, Luukko K, Poteryaev D, Laurikainen A, Sun YF, Laakso T, Eerikainen S, Tuominen R, Lakso M, Rauvala H, Arumae U, Pasternack M, Saarma M, Airaksinen MS (1999) Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor. Neuron 22:243-252.

Rusin KI, Moises HC (1995) mu-Opioid receptor activation reduces multiple components of high-threshold calcium current in rat sensory neurons. J Neurosci 15:4315-4327.

Rylett RJ, Williams LR (1994) Role of neurotrophins in cholinergic-neurone function in the adult and aged CNS. Trends Neurosci 17:486-490.

Saarma M, Sariola H (1999) Other neurotrophic factors: glial cell line-derived neurotrophic factor (GDNF). Microsc Res Tech 45:292-302.

Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. Br J Pharmacol 115:1265-1275.

Salo PT, Tatton WG (1993) Age-related loss of knee joint afferents in mice. J Neurosci Res 35:664-677.

Sariola H, Saarma M (2003) Novel functions and signalling pathways for GDNF. J Cell Sci 116:3855-3862.

Sarkar D, Fisher PB (2006) Molecular mechanisms of aging-associated inflammation. Cancer Lett 236:13-23.

Savidge JR, Ranasinghe SP, Rang HP (2001) Comparison of intracellular calcium signals evoked by heat and capsaicin in cultured rat dorsal root ganglion neurons and in a cell line expressing the rat vanilloid receptor, VR1. Neuroscience 102:177-184.

Schanne FA, Kane AB, Young EE, Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. Science 206:700-702.

Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA (1990) Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 75:40-47.

Schmelz M, Schmid R, Handwerker HO, Torebjork HE (2000) Encoding of burning pain from capsaicin-treated human skin in two categories of unmyelinated nerve fibres. Brain 123 Pt 3:560-571.

Schmidt R, Schmelz M, Torebjork HE, Handwerker HO (2000) Mechano-insensitive nociceptors encode pain evoked by tonic pressure to human skin. Neuroscience 98:793-800.

Scott B, Leu J, Cinader B (1988) Effects of aging on neuronal electrical membrane properties. Mech Ageing Dev 44:203-214.

Scott SA, Liang S, Weingartner JA, Crutcher KA (1994) Increased NGF-like activity in young but not aged rat hippocampus after septal lesions. Neurobiol Aging 15:337-346.

Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A 91:10771-10778.

Shishkin V, Potapenko E, Kostyuk E, Girnyk O, Voitenko N, Kostyuk P (2002) Role of mitochondria in intracellular calcium signaling in primary and secondary sensory neurones of rats. Cell Calcium 32:121-130.

Simonetti M, Fabbro A, D'Arco M, Zweyer M, Nistri A, Giniatullin R, Fabbretti E (2006) Comparison of P2X and TRPV1 receptors in ganglia or primary culture of trigeminal neurons and their modulation by NGF or serotonin. Mol Pain 2:11.

Slugg RM, Meyer RA, Campbell JN (2000) Response of cutaneous A- and C-fiber nociceptors in the monkey to controlled-force stimuli. J Neurophysiol 83:2179-2191.

Smith DE, Roberts J, Gage FH, Tuszynski MH (1999) Age-associated neuronal atrophy occurs in the primate brain and is reversible by growth factor gene therapy. Proc Natl Acad Sci U S A 96:10893-10898.

Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L, Egerton J, Charles KJ, Smart D, Randall AD, Anand P, Davis JB (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. Nature 418:186-190.

Smith MA (1996) Hippocampal vulnerability to stress and aging: possible role of neurotrophic factors. Behav Brain Res 78:25-36.

Smith MA, Cizza G (1996) Stress-induced changes in brain-derived neurotrophic factor expression are attenuated in aged Fischer 344/N rats. Neurobiol Aging 17:859-864.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.

Spray DC (1986) Cutaneous temperature receptors. Annu Rev Physiol 48:625-638.

Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol 2:695-702.

Stucky CL, Rossi J, Airaksinen MS, Lewin GR (2002) GFR alpha2/neurturin signalling regulates noxious heat transduction in isolectin B4-binding mouse sensory neurons. J Physiol 545:43-50.

Suzuki M, Mizuno A, Kodaira K, Imai M (2003a) Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 278:22664-22668.

Suzuki M, Watanabe Y, Oyama Y, Mizuno A, Kusano E, Hirao A, Ookawara S (2003b) Localization of mechanosensitive channel TRPV4 in mouse skin. Neurosci Lett 353:189-192.

Thibault O, Landfield PW (1996) Increase in single L-type calcium channels in hippocampal neurons during aging. Science 272:1017-1020.

Thibault O, Porter NM, Chen KC, Blalock EM, Kaminker PG, Clodfelter GV, Brewer LD, Landfield PW (1998) Calcium dysregulation in neuronal aging and Alzheimer's disease: history and new directions. Cell Calcium 24:417-433.

Tillman DB, Treede RD, Meyer RA, Campbell JN (1995a) Response of C fibre nociceptors in the anaesthetized monkey to heat stimuli: estimates of receptor depth and threshold. J Physiol 485 (Pt 3):753-765.

Tillman DB, Treede RD, Meyer RA, Campbell JN (1995b) Response of C fibre nociceptors in the anaesthetized monkey to heat stimuli: correlation with pain threshold in humans. J Physiol 485 (Pt 3):767-774.

Toescu EC (2000) Mitochondria and Ca(2+) signaling. J Cell Mol Med 4:164-175.

Toescu EC, Verkhratsky A (2000) Parameters of calcium homeostasis in normal neuronal ageing. J Anat 197 Pt 4:563-569.

Toescu EC, Verkhratsky A (2003) Neuronal ageing from an intraneuronal perspective: roles of endoplasmic reticulum and mitochondria. Cell Calcium 34:311-323.

Toescu EC, Verkhratsky A (2004) Ca2+ and mitochondria as substrates for deficits in synaptic plasticity in normal brain ageing. J Cell Mol Med 8:181-190.

Toescu EC, Myronova N, Verkhratsky A (2000) Age-related structural and functional changes of brain mitochondria. Cell Calcium 28:329-338.

Toescu EC, Verkhratsky A, Landfield PW (2004) Ca2+ regulation and gene expression in normal brain aging. Trends Neurosci 27:614-620.

Tominaga M, Tominaga T (2005) Structure and function of TRPV1. Pflugers Arch 451:143-150.

Tominaga M, Wada M, Masu M (2001) Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proc Natl Acad Sci U S A 98:6951-6956.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531-543.

Treede RD, Meyer RA, Campbell JN (1998) Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. J Neurophysiol 80:1082-1093.

Ulfhak B, Bergman E, Fundin BT (2002) Impairment of peripheral sensory innervation in senescence. Auton Neurosci 96:43-49.

Vanden Berghe P, Kenyon JL, Smith TK (2002) Mitochondrial Ca2+ uptake regulates the excitability of myenteric neurons. J Neurosci 22:6962-6971.

Vanterpool CK, Pearce WJ, Buchholz JN (2005) Advancing age alters rapid and spontaneous refilling of caffeine-sensitive calcium stores in sympathetic superior cervical ganglion cells. J Appl Physiol 99:963-971.

Verdu E, Ceballos D, Vilches JJ, Navarro X (2000) Influence of aging on peripheral nerve function and regeneration. J Peripher Nerv Syst 5:191-208.

Vilches JJ, Ceballos D, Verdu E, Navarro X (2002) Changes in mouse sudomotor function and sweat gland innervation with ageing. Auton Neurosci 95:80-87.

Voilley N, de Weille J, Mamet J, Lazdunski M (2001) Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. J Neurosci 21:8026-8033.

Waldmann R, Bassilana F, de Weille J, Champigny G, Heurteaux C, Lazdunski M (1997) Molecular cloning of a non-inactivating proton-gated Na+ channel specific for sensory neurons. J Biol Chem 272:20975-20978.

Walker KM, Urban L, Medhurst SJ, Patel S, Panesar M, Fox AJ, McIntyre P (2003) The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. J Pharmacol Exp Ther 304:56-62.

Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B (2002) Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. J Biol Chem 277:47044-47051.

Watkins LR, Maier SF (2002) Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev 82:981-1011.

Waxman SG (1999) The molecular pathophysiology of pain: abnormal expression of sodium channel genes and its contributions to hyperexcitability of primary sensory neurons. Pain Suppl 6:S133-140.

Waxman SG, Cummins TR, Dib-Hajj S, Fjell J, Black JA (1999) Sodium channels, excitability of primary sensory neurons, and the molecular basis of pain. Muscle Nerve 22:1177-1187.

Winter J, Forbes CA, Sternberg J, Lindsay RM (1988) Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. Neuron 1:973-981.

Witting N, Svensson P, Arendt-Nielsen L, Jensen TS (2000) Repetitive intradermal capsaicin: differential effect on pain and areas of allodynia and punctate hyperalgesia. Somatosens Mot Res 17:5-12.

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24:6410-6415.

Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S (1997) Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol 121:417-424.

Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-331.

Xiong J, Verkhratsky A, Toescu EC (2002) Changes in mitochondrial status associated with altered Ca2+ homeostasis in aged cerebellar granule neurons in brain slices. J Neurosci 22:10761-10771.

Xu GY, Huang LY (2002) Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. J Neurosci 22:93-102.

Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y, DiStefano PS, Curtis R, Clapham DE (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature 418:181-186.

Yaffe K, Lindquist K, Penninx BW, Simonsick EM, Pahor M, Kritchevsky S, Launer L, Kuller L, Rubin S, Harris T (2003) Inflammatory markers and cognition in well-functioning African-American and white elders. Neurology 61:76-80.

Yang C, Hutto D, Sah DW (2006) Distribution of GDNF family receptor alpha3 and RET in rat and human non-neural tissues. J Mol Histol 37:69-77.

Yurek DM, Fletcher-Turner A (2000) Lesion-induced increase of BDNF is greater in the striatum of young versus old rat brain. Exp Neurol 161:392-396.

Zhang RX, Lao L, Qiao JT, Ruda MA (2004) Effects of aging on hyperalgesia and spinal dynorphin expression in rats with peripheral inflammation. Brain Res 999:135-141.

Zhang X, Huang J, McNaughton PA (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. Embo J 24:4211-4223.

Zhu M, Oishi K, Lee SC, Patterson PH (2001) Studies using leukemia inhibitory factor (LIF) knockout mice and a LIF adenoviral vector demonstrate a key anti-inflammatory role for this cytokine in cutaneous inflammation. J Immunol 166:2049-2054.

Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002) Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J Neurosci 22:4057-4065.

Zwick M, Molliver DC, Lindsay J, Fairbanks CA, Sengoku T, Albers KM, Davis BM (2003) Transgenic mice possessing increased numbers of nociceptors do not exhibit increased behavioral sensitivity in models of inflammatory and neuropathic pain. Pain 106:491-500.